Interactions of phenolic compounds with globular proteins and their effects on food-related functional properties

Promotoren:
Prof. Dr. Ir. A.G.J. Voragen
Hoogleraar in de Levensmiddelenchemie

Prof. Dr. Ir. H. Gruppen
Persoonlijk hoogleraar bij de leerstoelgroep Levensmiddelenchemie

Co-promotor:
Dr. G. A. van Koningsveld
Toegevoegd onderzoeker, leerstoelgroep Levensmiddelenchemie

Promotiecommissie:
Prof. Dr. E.J.R. Sudhölter
Wageningen Universiteit

Dr. C.M.G.C. Renard
INRA, Le Rheu, France

Dr. H.M. Rawel
Universität Potsdam, Germany

Dr. W.J.H. van Berkel
Wageningen Universiteit

Dit onderzoek is uitgevoerd binnen de onderzoekschool VLAG (Voeding, Levensmiddelentechnologie, Agrobiotechnologie en Gezondheid).

Interactions of phenolic compounds with globular proteins and their effects on food-related functional properties

Stéphanie Prigent

Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
Prof. Dr. M. J. Kropff
in het openbaar te verdedigen
op maandag 12 september 2005
des namiddags te half twee in de Aula

ISBN: 90-8504-267-4

If you want to be happy, be.

Leo Tolstoï

Abstract

Stéphanie Prigent (2005). Interactions of phenolic compounds with globular proteins and their effects on food-related functional properties. Ph.D. thesis, Wageningen University, The Netherlands.

In order to modulate the functional properties of food proteins, the interactions between globular proteins and the monomeric phenolic, caffeoylquinic acid (CQA, chlorogenic acid), and the oligomeric phenolics, procyanidins, were characterized and investigated for their effect on protein functional properties.

Non-covalent interactions between proteins and CQA, as a representative for monomeric phenolics, involved a low affinity and did not affect protein solubility. Proteins show a medium affinity for procyanidins of an average degree of polymerization (DP) of 5.5, but weakly interacted with smaller procyanidins. Procyanidins of DP 5.5 strongly decreased protein solubility. Covalent interactions between proteins and CQA oxidised by polyphenol oxidase (PPO) or oxidised at alkaline pH resulted in protein modification mainly via dimeric CQA quinones. The covalent modifications of proteins with CQA strongly reduced protein solubility at pH $\leq$ pI and induced some protein dimerisation. The observations on covalently modified proteins were clarified by studying amino-acid side-chain reactivity. Masses corresponding to a dimer of CQA quinone bound to lysine, tyrosine, histidine and tryptophan, and to a monomer of CQA quinone bound to histidine and tryptophan were observed. Lysine and tyrosine were more reactive than histidine. Tryptophan showed a lower reactivity.

It can be concluded that for food non-covalent interactions are restricted to oligomeric phenolic, despite the presence of relatively large amount of monomeric phenolics. For covalent interactions, the interaction is not restricted to lysine and cysteine, but also tyrosine, histidine, and to a lesser extent tryptophan interact.

Symbols and Abbreviations

ADT	Automatic drop tensiometer
ALK	Samples modified under alkaline conditions
Boc	Tert-butyloxycarbonyl group
BSA	Bovine serum albumin
CD	Circular dichroism
CE	Capillary electrophoresis
cIEF	Capillary isoelectric focusing
CQA	Caffeoylquinic acid (chlorogenic acid)
Da	Dalton
DOPA	Dihydroxyphenylalanine
DP	Degree of polymerisation
DSC	Differential scanning calorimetry
EDTA	Disodium ethylenediamine tetra-acetate
ESI	Electrospray ionisation
HD-GF	method of Hummel and Dreyer using gel filtration
I	Ionic strength
ITC	Isothermal titration calorimetry
JR	Jeanne Renard variety
k_i	Binding constant
LC-MS	Liquid chromatography - mass spectrometry
LCQ	Liquid chromatography quadrupole
MALDI-TOF	Matrix assisted laser desorption ionization time-of-flight
MM	Marie Ménard variety
MS	Mass spectrometry
MW	Molecular weight
m/z	Mass/charge
ν	Number of CQA molecules bound per protein molecule
n_i	Number of binding sites
NMR	Nuclear magnetic resonance
OPA	Orthophtaldialdehyde
PAG	Primary amino groups
PC	Phenolic compounds
pI	Isoelectric point
POD	Peroxydase
PP	Samples incubated with polyphenol oxydase
PPO	Polyphenol oxydase
PRP	Proline-rich proteins
RP-HPLC	Reverse-phase high-performance liquid chromatography
RT	Retention time
SDS-PAGE	Sodium dodecyl sulfate – polyacrylamide gel electrophoresis
SPR	Surface plasmon resonance
TFA	Trifluoroacetic acid
TIC	Total ion count
Tm	Phase transition temperature

CONTENTS

Chapter 1

General introduction

1. PHENOLIC COMPOUNDS: INTRODUCTION AND DEFINITION

Plant phenolic compounds, also denoted polyphenols, are defined as compounds possessing one or more aromatic rings bearing hydroxyl substituent(s), and which are derived from the secondary metabolism of plants (Parr and Bolwell, 2000;Robards et al., 1999). The term "polyphenols" may also refer to synthetic compounds used in the chemical industry for coating and painting (Handique and Baruah, 2002). But in the food industry, the term predominantly refers to plant phenolic compounds that are present in foods and in the plant raw materials used in food industry.

In plants, phenolic compounds play a role in numerous processes, such as plant growth and reactions to stress and pathogen attack (Parr and Bolwell, 2000). Plant phenolic compounds are present in products ranging from food to sunblockers and paper. As an example, the yellowing of paper over the years is caused by photochemical reactions of phenolic compounds (Zhu and Gray, 1995). Phenolic compounds can be found in many foods and drinks from plant origin, e.g. fruits, vegetables, coffee (Clifford, 1999), tea (Lakenbrink et al., 2000), beer, wine and chocolate (Arts et al., 1999). Red wine for example has a total content of phenolic compounds of 1-4 g/L (Shahidi and Naczk, 1995). These high amounts have led to the hypothesis that phenolic compounds, especially resveratrol from the stilbene sub-class, would be one of the responsible factors for the beneficial effect of wine consumption on cardiovascular diseases (Wallerath et al., 2005). As another example, dark chocolate contains approximately 1.6 g/kg of oligomeric phenolic compounds, called proanthocyanidins (USDA database, 2004), while a member of the proanthocyanidin sub-class, the procyanidins, is present in particularly high concentrations in apples and cider (2-3 g/L) (Shahidi and Naczk, 1995). The content of phenolic compounds in foods may change during storage due to effects induced by light and temperature (Friedman, 1997). Apart from being naturally present in the raw materials used for foods, phenolic compounds are also added to some foods for their coloring properties and for their antioxidant effects (O'Connell and Fox, 2001;Richelle et al., 2001).

In foods, the presence of phenolic compounds may be easily observable due to the chromophoric groups that some phenolic compounds bear, e.g. the red-purple anthocyanins (Bakowska et al., 2003), or by the brown and green reaction products of phenolic compounds

with themselves or with proteins (Montavon et al., 2003;Yabuta et al., 2001). This browning can be easily observed when fruits are damaged (Richard-Forget and Gauillard, 1997;Robards et al., 1999). The presence of phenolic compounds can also affect the taste of food, as low concentrations of phenolic compounds may be responsible for desirable sweet, smoky or caramel flavours in e.g. dairy products (O'Connell and Fox, 2001). One can also notice the high concentrations of phenolic compounds in tea and wine by the astringent sensation they give. The latter results from the precipitation of saliva proteins on the tongue by interactions with specific phenolic compounds (Baxter et al., 1997;Charlton et al., 2002). If milk is added to tea, the proteins present in milk will bind most of the present phenolic compounds, leaving the saliva proteins unaffected. On the other hand, the interactions between phenolic compounds and proteins may lead to a decrease of protein digestibility, by blocking the substrate and/or inhibiting certain proteases (Kroll et al., 2003).

The interactions between phenolic compounds and proteins also play a role in the processing of certain food products. For example, the binding of proteins to immobilized proanthocyanidins is used to clarify wine (Ferreira et al., 2002). In beer and fruit juices, certain undesirable hazes contain phenolic compounds and proteins (Beveridge et al., 1996), which suggests that phenolic compound-protein interactions play a role in haze formation (Siebert, 1999). Recently, covalent interactions between proteins and caffeic acid were shown to improve network formation in gelatin gels, giving them a greater mechanical strength and a higher thermal stability (Strauss and Gibson, 2004). The interactions between phenolic compounds and proteins have also consequences for the production of plant protein ingredients, as these interactions may hinder protein extraction. Removing phenolic compounds for example is one of the main issues for the production of protein products from sunflower (Gonzalez-Perez et al., 2002) and phenolic compounds may be responsible for the low solubility of some potato protein preparations (Van Koningsveld et al., 2002). Because protein insolubility also hinders other protein functional properties, protein solubility is an important factor in protein functionality.

A better understanding of phenolic compound-protein interactions would help to control the functional properties of proteins in food products and the production of protein ingredients.

1.1 Classification of phenolics

Phenolic compounds represent a wide range of molecules with a molecular mass from about 100 to 3,000-4,000 Da, although the maximum molecular mass has not been clearly defined

(Haslam, 1996). They are produced by two principal pathways: the shikimate and the polyketide pathway (O'Connell and Fox, 2001). Different classifications of phenolic compounds have been proposed (O'Connell and Fox, 2001). For example, plant "tannins" are proposed to be broadly divisible into two majors groups: the proanthocyanidins and the polyesters based on gallic and/or hexahydroxydiphenic acid (Haslam, 1989). However, this classification does not include, for example, simple phenols. In **Figure 1**, a more complete classification according to the number of carbon atoms is given. It is adapted from the one given by O'Connell and Fox (O'Connell and Fox, 2001) and divides the phenolic compounds into five groups: 1) the C_6 group, comprising simple phenols and benzoquinones; 2) the C_6C_n group, which includes phenolic acid derivatives and hydroxycinnamic acid derivatives (**Figure 2**); 3) the C_6-C_n-C_6 group, which includes flavanoids (C_6-C_3-C_6); 4) the $(C_6$-$C_3)_n$, group consisting of lignans and lignins; and finally 5) the tannin group, which are divided into hydrolysable tannins and condensed tannins (O'Connell and Fox, 2001). The hydrolysable tannins are formed by gallic acid, 3-digallic acid or hexahydroxydiphenic acid, esterified to a polyol such as glucose or quinic acid (O'Connell and Fox, 2001). The condensed tannins mainly consist of the sub-class of the proanthocyanidins, as will be explained later (O'Connell and Fox, 2001). Proanthocyanidins are oligomers and polymers of flavanols (**Figure 2**), which are members of the flavanoid sub-class (O'Connell and Fox, 2001). It has to be noted that, therefore, some authors classify proanthocyanidins in the same class as their monomeric units, i.e. the C_6-C_n-C_6 class. Among these monomeric units, (+)-catechin, (-)-epicatechin, (+)-gallocatechin and (-)-epigallocatechin are the most common (Haslam, 1989). Their 2S enantiomers, e.g. (-)-catechin and (+)-epicatechin, may also be present (Haslam, 1989).

During the studies described in this thesis, representatives of two classes of phenolic compounds that occur commonly in foods will be considered: 5'-caffeoylquinic acid, as a representative of the hydroxycinnamic acids, which belong to the C_6C_n class, and proanthocyanidins as a representative of the condensed tannins.

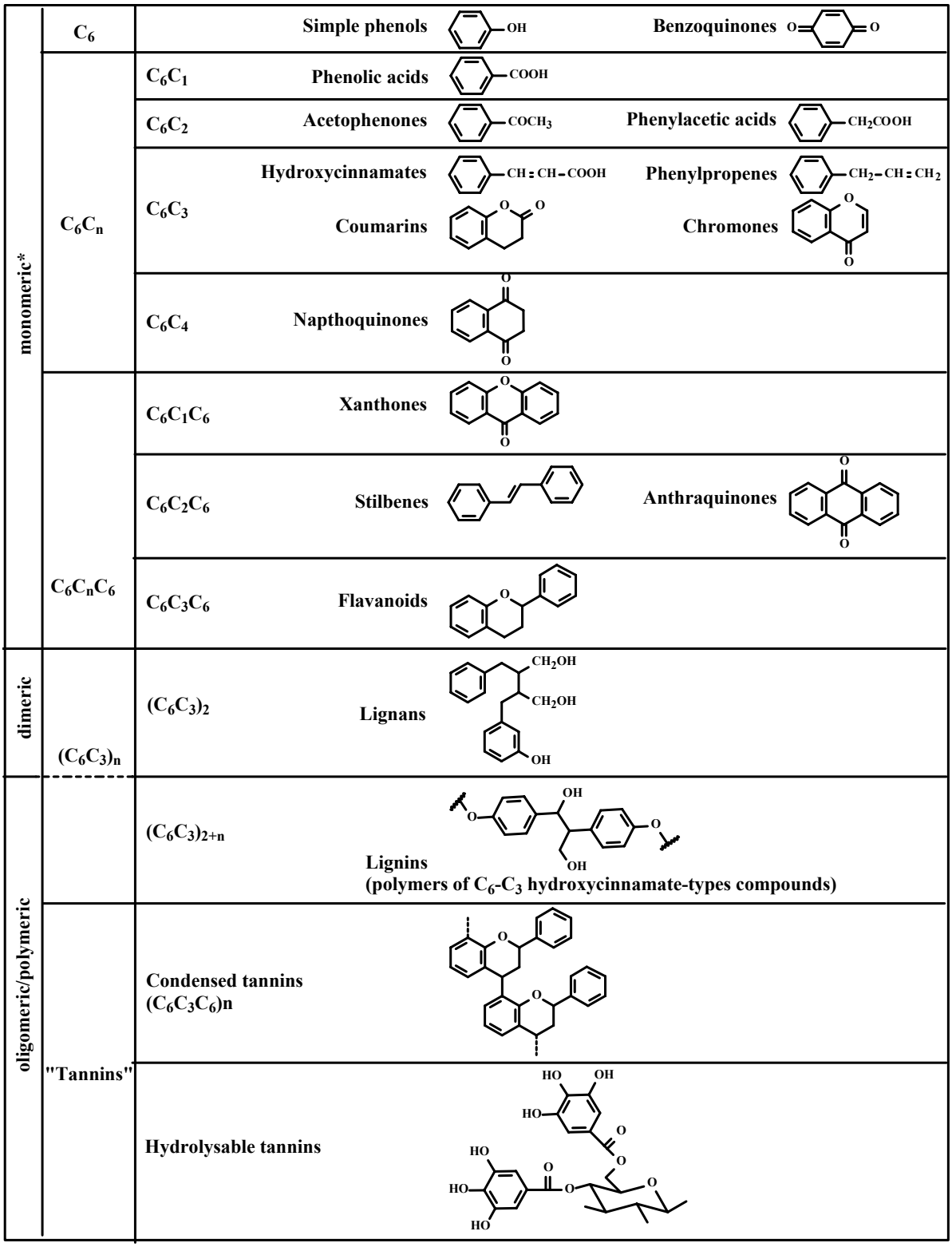
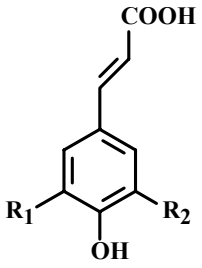
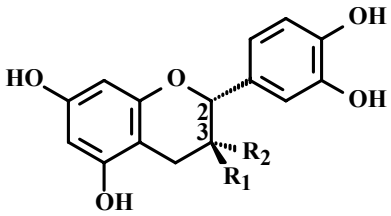


Figure 1. Classification of phenolic compounds, adapted from O'Connell and Fox (O'Connell and Fox, 2001)

* Oligomers are occasionally formed in these groups.





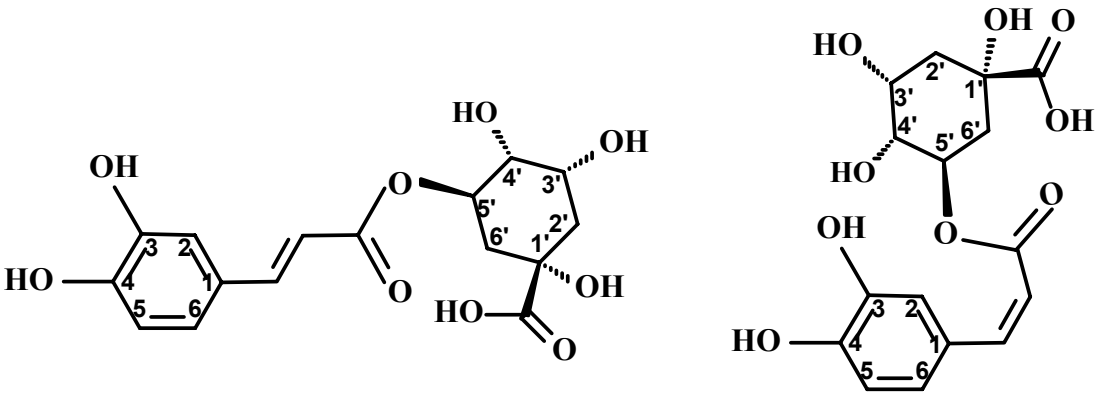
p-Coumaric acid: R_1 = H ; R_2 = H
Caffeic acid: R_1 = OH ; R_2 = H
Ferulic acid: R_1 = OCH_3 ; R_2 = H
Sinapic acid: R_1 = OCH_3 ; R_2 = OCH_3

(+)-Catechin: R_1 = OH; R_2 = H
(-)-Epicatechin: R_1 = H ; R_2 = OH

Figure 2. Structure of some hydroxycinnamic acids and flavanols

1.2 Caffeoylquinic acid

5'-Caffeoylquinic acid (CQA; 1',3',4',5'-tetrahydroxycyclohexanecarboxylic acid 3-(3,4)-dihydroxypropenyldihydroxyphenyl-1-propenoate, **Figure 3**) is composed of a molecule of caffeic acid esterified with quinic acid (Clifford, 2000). Its molecular mass is 354 Da. Caffeic acid and its esters are usually the predominant hydroxycinnamic acids in fruits (Shahidi and Naczk, 1995). Caffeoylquinic acid is present in high concentrations in certain foods and is considered to be the main phenolic compound responsible for browning (Shahidi and Naczk, 1995). Because of its high concentration in some foods (up to 700 mg per cup of coffee (Clifford, 1999) and its ubiquitous character, CQA is often chosen as a model compound for studying hydroxycinnamic acid derivatives and the simple phenolic compounds. CQA is often referred to as "chlorogenic acid" (Clifford, 2000), although the chlorogenic acids are actually a group of compounds, that contains also 3'-caffeoylquinic acid ("neochlorogenic acid") and 4'-caffeoylquinic acid ("cryptochlorogenic acid"). The most common caffeoylquinic acids are 5'- and 3'-caffeoylquinic acid (Mathew and Lakshminarayana, 1969). 5'-Caffeoylquinic acid exists as a trans and a cis isomer (Mathew and Lakshminarayana, 1969). The compound used in this study is the trans isomer, which is the most abundant in nature. Additionally, depending of the position of the hydroxyl group in the quinic acid moiety, several R and S configurations can be encountered (Haribal et al., 1998). Whereas CQA can be oxidised at alkaline pH, it is stable at acidic pH at moderate temperatures (Friedman and Jürgens, 2000).



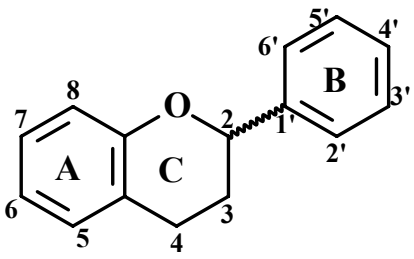
trans-5'-caffeoylquinic acid **cis-5'-caffeoylquinic acid**

Figure 3. Structure of 5'-caffeoylquinic acid (CQA)

1.3 Condensed tannins

The most common sub-class of condensed tannins in food is the proanthocyanidins (Haslam, 1989). The term "proanthocyanidins" is derived from the fact that they are cleaved into anthocyanidins upon heat treatment at acidic pH (Swain and Hillis, 1959). Proanthocyanidins can be classified based on their hydroxylation pattern. They are always hydroxylated at the 3, 5 and 7 position, while hydroxylation on the B ring varies. The most common members of the proanthocyanidins are the procyanidins, formed from catechin units (**Figure 4**), and the prodelphinidins, formed from gallocatechin units (USDA database, 2004;Haslam, 1989;Shahidi and Naczk, 1995). Having the structure of a catechin molecule that is dehydroxylated at the 3'-position, afzelechin is the sub-unit of propelargonidins (**Figure 4**), which are a rare member of the proanthocyanidins (Haslam, 1989). In addition to these proanthocyanidins, other members of condensed tannins exist and are usually not called proanthocyanidins (Hemingway, 1989). These condensed tannins have different hydroxylation patterns and are only rarely encountered. In contrast to the proanthocyanidins, these condensed tannins are dehydroxylated on their 5-position (proguibourtinidins, profisetinidins, prorobinetinidins) or on their 3-position (proapigeninidins, proluteolinidins) (Haslam, 1989)(**Figure 4**). Two additional, particularly rare, members exist: proteracacidins and promelacacidins, which are dehydroxylated on their 5-position and hydroxylated on their 8-position (Hemingway, 1989)(**Figure 4**). Proanthocyanidins are difficult to analyse directly and to quantify in foods, because of their multimeric and polydisperse nature (Santos-Buelga and Scalbert, 2000). In this study, procyanidins, which are the predominant proanthocyanidins

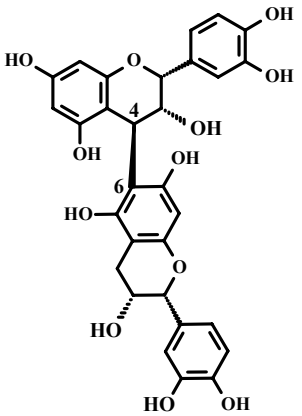
in fruits (Shahidi and Naczk, 1995), are used to study the interactions between oligomeric phenolic components and proteins.



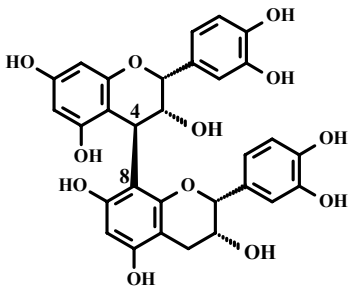
Members	Hydroxylation pattern	
Procyanidin	3, 3', 4', 5, 7	Proanthocyanidins
Prodelphinidin	3, 3', 4', 5, 5', 7	
Propelargonidin	3, 4', 5, 7	
Proguibourtinidin	3, 4', 7	
Profisetinidin	3, 3', 4', 7	
Prorobinetinidin	3, 3', 4', 5', 7	
Proteracacidin	3, 4', 7, 8	
Promelacacidin	3, 3', 4', 7, 8	
Proapigeninidin	4', 5, 7	
Proluteolinidin	3', 4', 5, 7	

Figure 4. Structure of the monomeric units of condensed tannins (adapted from Hemingway, 1989)

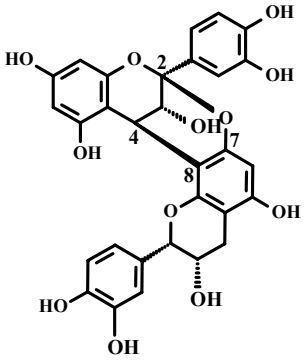
Proanthocyanidins are classified not only according to their constitutive units, but also according to the nature and number of interflavan bond(s), and according to their degree of polymerization. The consecutive units of proanthocyanidins are linked through interflavan bond(s), usually between C4 and C6 or between C4 and C8 (Shahidi and Naczk, 1995). Double linkages, which occasionally occur, are between C4-C6 or C4-C8 and C2-O-C5 or C2-O-C7. This linkage may be an axial (**Figure 5**) or an equatorial bound. The degree of polymerization is symbolized by a letter, e.g. B = dimer, C = trimer, etc. The letter A is used to name the proanthocyanidins possessing double linkages. The letter is followed by an arbitrarily chosen number to indicate the stereochemistry of the proanthocyanidins.



procyanidin B5
epicatechin (4β -> 6)- epicatechin



procyanidin B2
epicatechin (4β -> 8)- epicatechin



procyanidin A2
epicatechin (2-> O-> 7, 4-> 8)- epicatechin

Figure 5. Structure of some dimeric proanthocyanidins

2. OXYDATION OF PHENOLIC COMPOUNDS

2.1 Formation of quinones

Ortho-diphenolic compounds can be oxidised into o-quinones (**Figure 6**). These quinones can be formed enzymatically or non-enzymatically. Non-enzymatic oxidation occurs readily at alkaline pH (Yabuta et al., 2001), as will be discussed later. It has been proposed that non-enzymatic oxidation could also be rather important at low pH during prolonged food storage (Cilliers and Singleton, 1989). However, the latter study was performed under continuous stirring and aeration, which is usually not the case during food storage. Therefore, it can be quite safely considered that non-enzymatic oxidation in food ingredients at low pH is limited.

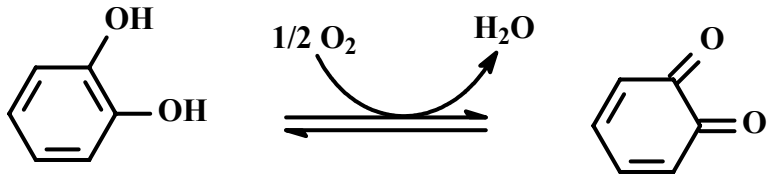


Figure 6. Oxydation of an o-diphenol into a quinone

- Enzymatic oxidation:

Quinones can be formed via the action of two types of enzymes: polyphenol oxidases and peroxidases. Quinones are produced by peroxidases via the formation of radicals. Peroxidases require the presence of hydrogen peroxide (Matheis and Whitaker, 1984), which thus makes their role in foods limited compared to the action of polyphenol oxidases, on which we will focus.

Polyphenol oxidases (EC 1.14.18.1) are divided into catechol oxidases and laccases. Both enzymes can oxidise phenolic substrates using molecular oxygen (Mayer and Harel, 1979;Osuga et al., 1994). Catechol oxidases can catalyse the oxidation of o-diphenols to o-quinones using their catecholase activity (Mayer and Harel, 1979) (**Figure 6)**. Furthermore, when they also possess the so-called cresolase activity, catechol oxidases may convert monophenols to o-diphenols (Mayer and Harel, 1979;Rodríguez-López et al., 2001) (**Figure 7)**. Laccases are able to oxidise a broader range of substrates than catechol oxidases, including *p*-diphenols (Mayer and Harel, 1979;Mayer and Staples, 2002) and non phenolic compounds e.g. phosphorothiolates (Amitai et al., 1998). They are also able to catalyze other reactions than oxidation, such as demethylation and (de-)polymerisation of phenolic compounds (Mayer, 1987).

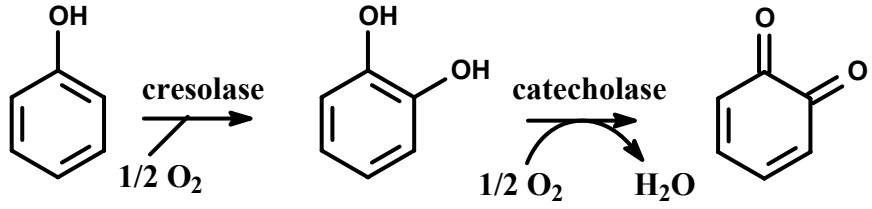


Figure 7. Cresolase and catecholase mechanisms of polyphenol oxidase. (adapted from Matheis and Whitaker, 1984)

Catechol oxidase is usually called tyrosinase in mammals and mushrooms (Sanoner, 2001). Tyrosinases from mammals are relatively specific for tyrosine and DOPA (dihydroxyphenylalanine), whereas catechol oxidases from fungi and higher plants show activity on a wider range of mono- and o-diphenols (Mayer and Harel, 1979). Mushroom tyrosinase from *Agaricus bisporus*, which is used in this thesis, is a copper-containing enzyme with a molecular weight around 110 kDa and a pI of 4.7-5.0 (Robb and Gutteridge, 1981). It is a tetramer formed from two subunits of 43 kDa and two subunits of 13 kDa (Robb and Gutteridge, 1981).

The pH optimum of most catechol oxidases is between pH 5.0 and pH 7.0. The activity of mushroom tyrosinase was reported to be optimal at pH 7.0 and to be negligible at pH 4.0 (McCord and Kilara, 1983). For a complete scheme of the conversion of phenols into quinones by PPO, the articles of Fenoll, Peñalver and co-workers should be consulted (Fenoll et al., 2001;Fenoll et al., 2002;Fenoll et al., 2004;Peñalver et al., 2002).

The action of polyphenoloxidase can be inhibited by removal of the oxygen or of the phenolic substrate. For example, addition of polyvinylpyrrolidone, a synthetic polyamide, which interacts with some phenols, makes them unavailable for the enzyme (Mayer and Harel, 1979). The enzyme can also be inactivated by the direct interaction with an inhibitor, e.g. EDTA, which binds to copper in the active site (Mayer and Harel, 1979). Another way of neutralizing the phenolic oxidation is the addition of reducing compounds, like vitamin C or certain thiol compounds, which revert the oxidised phenolic to the original compound (Negishi and Ozawa, 2000;Richard-Forget et al., 1992a).

- Non-enzymatic oxidation

The formation of quinones may also occur in the absence of an enzyme. Increasing the pH induces the deprotonation of the phenolic hydroxyl group, eventually leading to the formation of quinones. This method is used in food industry e.g. to produce black olives by treating

them with diluted NaOH in order to oxidise the caffeic acid and hydroxytyrosol present in olives (García et al., 1996). An additional possibility to form quinones is the use of an oxidising reagent such as e.g. periodate (Harrison and Hodge, 1982). This permits to study the formation of quinones, and its concomitant effects, also at acidic pH.

Quinones themselves are unstable compounds, which tend to react by an oxido-reduction mechanism with other molecules, by oligomerization with other phenolic compounds, or by covalent reactions with other molecules such as proteins.

2.2 Reactions of quinones with phenolic compounds

Quinones have the ability to oxidise other phenolic compounds via the oxido-reduction mechanism, depending on the redox potential of the phenolics (Cheynier et al., 1988). This coupled oxidation can thus induce the oxidation of compounds, which are otherwise not substrates for catechol oxidases.

The oxido-reduction mechanism is the main phenomenon at low pH, whereas at higher pH values quinones tend to covalently react with other phenolic molecules leading to formation of dimers (Cheynier et al., 1988;Richard-Forget et al., 1992b). Via this mechanism also higher molecular weight condensation products can be formed (Cheynier et al., 1988). The formation of dimers induced by oxidation of phenolic compounds was observed with gallic acid (Kawabata et al., 2002;Tulyathan et al., 1989) and caffeic acid (Cilliers and Singleton, 1991;Fulcrand et al., 1994;Rompel et al., 1999;Tazaki et al., 2001;Yabuta et al., 2001), CQA (Antolovich et al., 2004) and catechin (Guyot et al., 1996;Oszmianski and Lee, 1990). Various dimeric structures were formed upon oxidation of caffeic acid, as presented in **Figure 8** (Cilliers and Singleton, 1991;Fulcrand et al., 1994;Rompel et al., 1999;Tazaki et al., 2001;Yabuta et al., 2001).

CQA was also proven to dimerise (Antolovich et al., 2004), leading to the formation of various dimers as observed by reverse-phase chromatography (Bernillon et al., 2004). The structure of one of these CQA dimers has been elucidated and corresponds to the same structure as presented in **Figure 9** (personal communication from Bernillon, 2005). The structures of other CQA dimers have not been elucidated. Because the carboxylic acid group of caffeic acid is esterified to form CQA, dimers “A” and “B” in **Figure 8** cannot be formed with CQA.

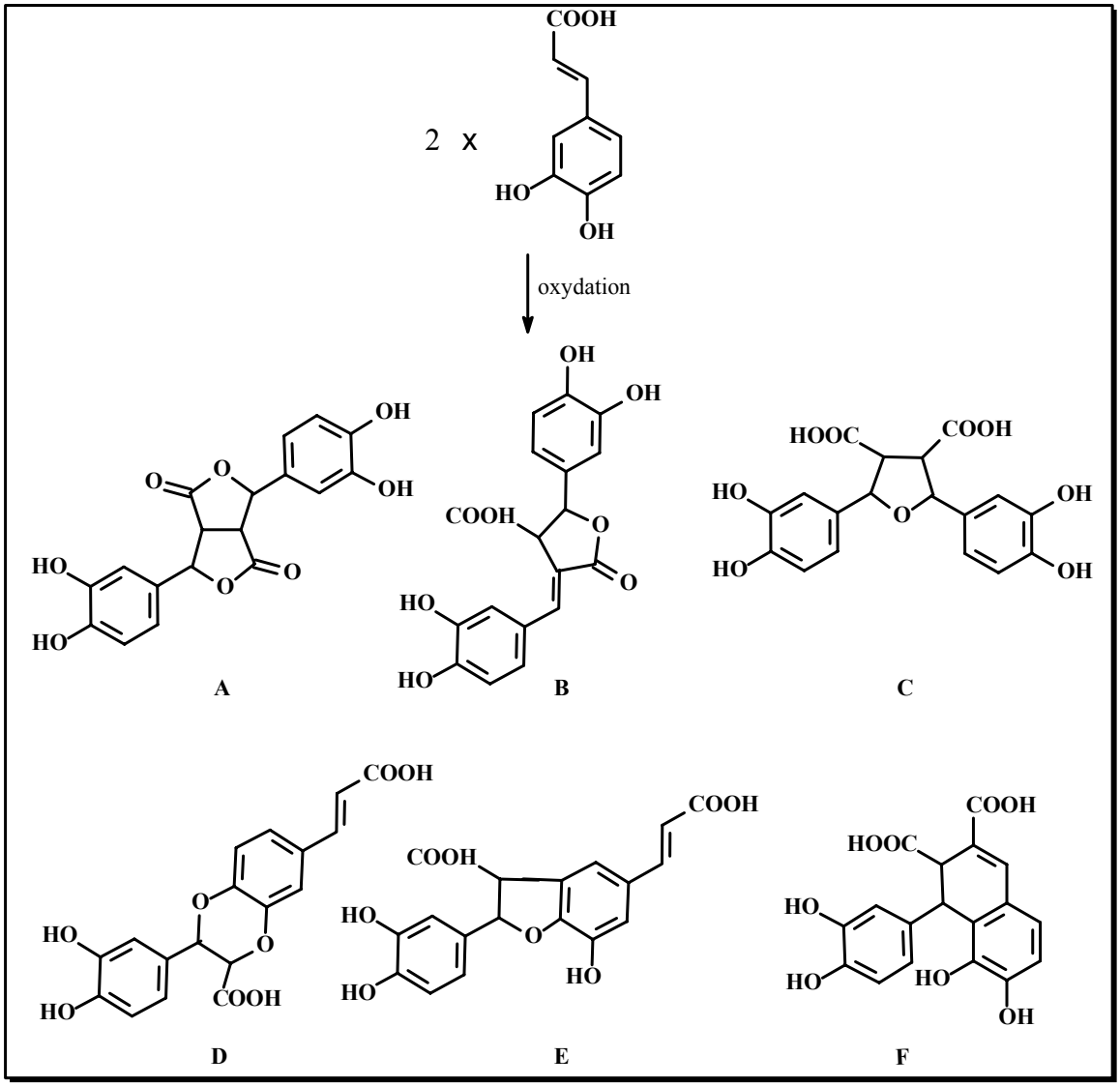


Figure 8. Dimers formed from caffeic acid oxydation (Cilliers and Singleton, 1991;Fulcrand et al., 1994;Rompel et al., 1999;Tazaki et al., 2001;Yabuta et al., 2001)

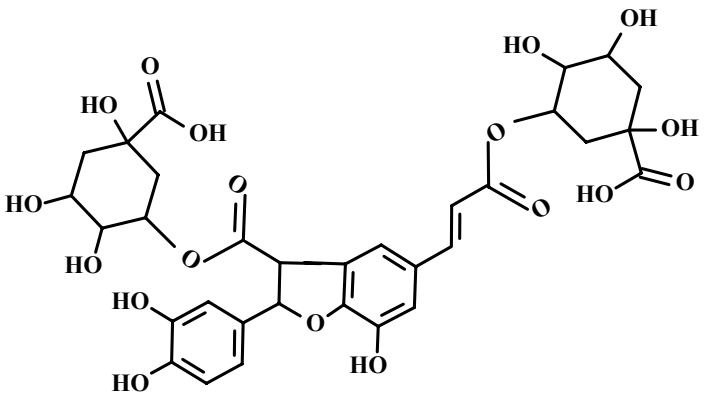


Figure 9. Dimer formed from CQA oxydation (personal communication from Bernillon, 2005).

An example of the proposed mechanism for the formation of one of the possible dimers of ethyl caffeate (skeleton similar to dimer "F" on **Figure 8**) is presented in **Figure 10** (Namiki et al., 2001;Yabuta et al., 2001). This dimerisation occurs via the binding of the isoprenyl groups of two molecules of ethyl caffeate (**Figure 10**, Yabuta et al., 2001).

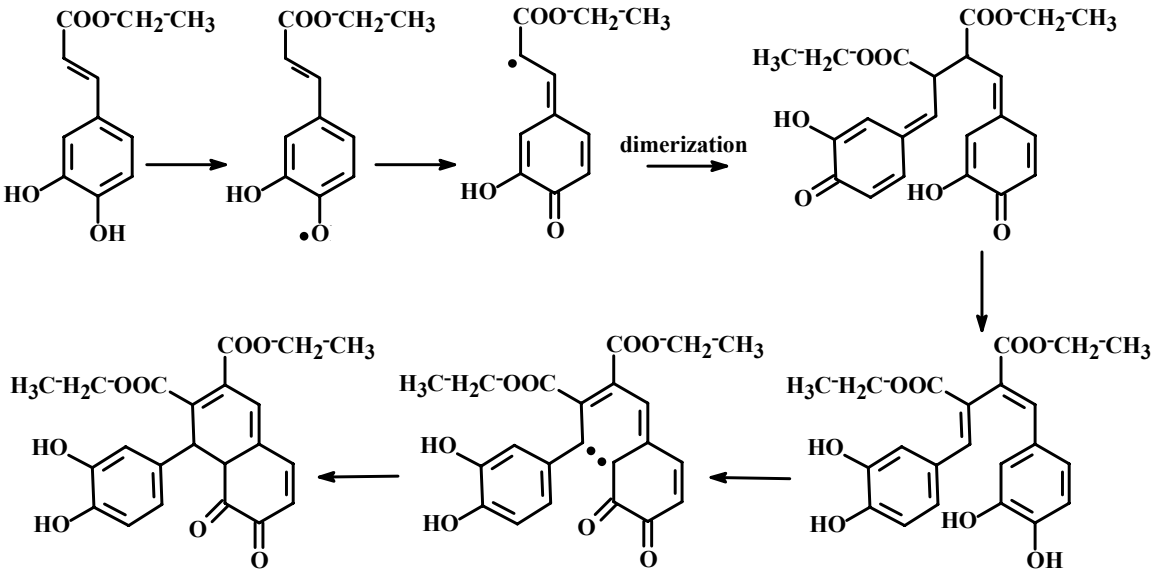


Figure 10. Proposed mechanism of dimerisation of ethyl caffeate at alkaline pH (Namiki et al., 2001; Yabuta et al., 2001)

3. NATURE OF PROTEIN-PHENOLIC INTERACTIONS

Phenolic compounds can interact with proteins in two different ways: via non-covalent (reversible) interactions and via covalent interactions, which in most cases are irreversible. Whatever the type of interactions, two types of complexation mechanisms can be distinguished: a monodentate and a multidentate mechanism (Haslam, 1989). "Monodentate" means that a phenolic compound interact with only one protein site. At a high phenolic compound to protein ratio, phenolics can form a layer around a protein molecule, thereby more or less covering its surface, via a monodentate mechanism (**Figure 11**). The layer at the surface of the protein makes it less hydrophilic, which may lead to aggregation. The other mechanism, the multidentate mechanism, applies only to phenolic compounds with sufficient size to be able to interact with more than one site, thus being able to form cross-links between proteins (**Figure 11**). Both complexation mechanisms may lead to aggregation and precipitation (Charlton et al., 2002;Haslam, 1989). The multidentate mechanism requires a much lower phenolic compound / protein molar ratio and thus a lower phenolic compound

concentration than the monodentate mechanism. Proanthocyanidins, therefore, would decrease protein solubility at much lower ratios than monomeric phenolic compounds, such as CQA.

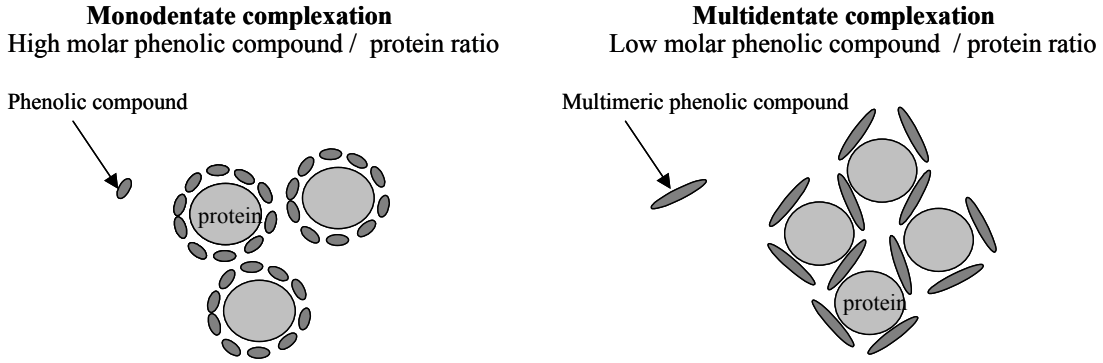


Figure 11. Monodentate and multidentate mechanism, adapted from Haslam, 1989

The non-covalent and covalent interactions between phenolic compounds and proteins do not only depend on the phenolic compound / protein ratio, but also on factors such as steric hindrance and the polarity of both the protein and the phenolic compound involved. Therefore, the nature and the sequence of amino acids residues in the protein chain are of particular importance. The mechanisms involved in the non-covalent and the covalent interactions will be explained in the following section.

3.1 Non-covalent interactions

Non-covalent interactions can be divided into five types: electrostatic interactions, van der Waals interactions, hydrogen bonds, hydrophobic interactions and π bonds. In the case of phenolic compound-protein interactions, hydrogen bonding and hydrophobic interactions are said to be the main driving forces. Hydrogen bonds may involve the interactions between hydroxyl groups of phenolic compounds and the nitrogen or oxygen of lysine, arginine, histidine, asparagine, glutamine, serine, threonine, aspartic acid, glutamic acid, tyrosine, cysteine and tryptophan. Hydrophobic interactions may occur between phenolic compounds and amino acids such as e.g. alanine, valine, isoleucine, leucine, methionine, phenylalanine, tyrosine, tryptophan, cysteine and glycine residues. Hydrogen bonding and hydrophobic interactions follow an opposite trend upon heating; an increase in temperature causes a decrease in hydrogen bonding, but an increase in hydrophobic interactions.

Two theories exist to explain the relative importance of hydrogen bonding and hydrophobic interactions between phenolic compounds and proteins. In the first hypothesis, two stages

occur: the associations are driven by hydrophobic interactions, after which hydrogen bonding enhances the interactions (Haslam, 1989). In the second hypothesis, the nature of the phenolic compound is considered: hydrophobic interactions are the main forces responsible for the interactions with nonpolar phenolic compounds such as pentagalloylglucose, whereas hydrogen bonding is the main force driving the interactions with more polar phenolic compounds such as procyanidins (Hagerman et al., 1998). This second hypothesis can explain the different effects exerted by heating on the interactions between gallotannins and proanthocyanidins. Heating increases the interaction between BSA and the nonpolar pentagalloylglucose, suggesting dominant hydrophobic interactions, but has no effect on the interactions with the more polar procyanidin dimer (Hagerman et al., 1998). The latter suggests a balance between hydrophobic and hydrophilic interactions. In conclusion, it seems that both hydrogen bonding and hydrophobic interactions are involved, while the nature of the phenolic compound, the protein and the environment determine which kind of interactions is the most important.

Considering the types of phenolic compounds, not only the polarity of the phenolic compound influences the binding, but also the size and the flexibility of the phenolic compound. The larger the phenolic compound, or more exactly the more binding sites the phenolic compound possesses, the stronger the association (Hagerman et al., 1998), i.e. proanthocyanidin trimers bind more tightly to BSA than proanthocyanidin dimers (Artz et al., 1987). However, these interactions do have an optimum as less protein is precipitated with procyanidins > 3.5 kDa than with smaller procyanidins (De Freitas and Mateus, 2001a). The lower solubility of large phenolic compounds may explain why phenolic compounds above a certain size do not easily interact with proteins (De Freitas and Mateus, 2001a). In addition to this, the size of the phenolic compound can decrease its conformational flexibility, which is observed to be an important parameter in protein-phenolic compound interactions (Frazier et al., 2003).

With respect to the type of protein, it has been shown that globular proteins, which are small and compact, have a lower affinity for phenolic compound than proteins with a more open conformation, e.g. proline-rich proteins (PRP), such as collagen. When a proline residue interacts with a phenolic compound, a specific interaction takes place: this binding is controlled by weak forces occurring at short distances between the aromatic groups of phenolic compounds and proline (Bianco et al., 1997). Such interactions may also occur between aromatic groups of two phenolics (Baxter et al., 1997).

3.2 Covalent interactions

Covalent interactions between phenolic compounds and proteins can occur via oxidation of phenolic compounds to radicals or quinones. Since, as explained previously, the formation of radicals by peroxidase in food and its raw materials is limited, only the reaction between quinones and proteins is described below. In addition to this mechanism, also the reaction of a protein with a carbocation derived from proanthocyanidin degradation is a theoretical possibility for occurrence of protein-phenolic compound interactions.

3.2.1 Covalent interactions of quinones with proteins and other nucleophilic molecules

Quinones have been indirectly proven to react with amino acids incorporated in a peptide chain. The terminal amino group of proteins (Pierpoint, 1969a;Pierpoint, 1969b), as well as cysteine (Felton et al., 1989), lysine (Pierpoint, 1982;Rawel et al., 2000), tryptophan (Rawel et al., 2002) and histidine (Hurrell et al., 1982) side-chains have been shown, by analysis of the amino acid content and by measurements of the amount of non-reacted free amino acids, to react with quinones. Direct evidence for reaction products with the side-chains of amino acids has been demonstrated by the covalent reaction between CQA and cysteine (Pierpoint, 1966;Richard et al., 1991), caffeic acid and cysteine (Cilliers and Singleton, 1990), oxidised catechols and histidine (Kerwin et al., 1999;Kramer et al., 2001;Xu et al., 1996) and oxidised catechols and methionine (Vithayathil and Satyanarayana Murthy, 1972).

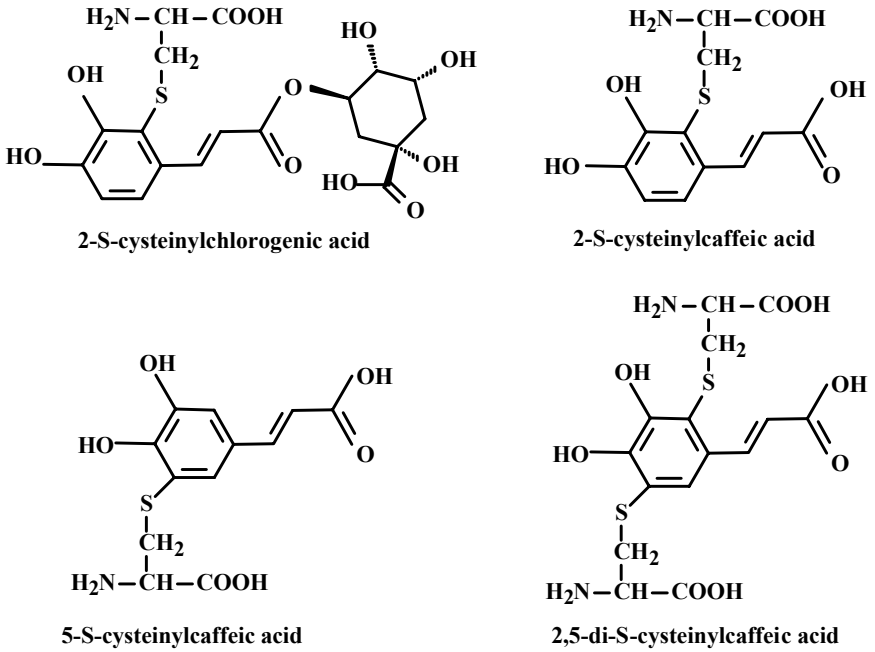


Figure 12. Reaction products of cysteine and oxidised CQA and oxidised caffeic acid (Cilliers and Singleton, 1990;Richard et al., 1991)

The reaction between CQA and cysteine leads to the formation of 2-S-cysteinylchlorogenic acid (Richard et al., 1991) (**Figure 12**). The 2-position of the aromatic ring of oxidised CQA is the most electrophilic position and, therefore, it is the most susceptible site to react with nucleophiles, such as cysteine (Cilliers and Singleton, 1990). The reaction between caffeic acid and cysteine occurs also at this position. In addition, the 5-position of oxidised caffeic acid was found to be able to react with cysteine yielding to 5-S-cysteinylcaffeic acid and may even result in the binding of two cysteine molecules resulting in the formation of 2,5-di-S-cysteinylcaffeic acid (Cilliers and Singleton, 1990). A reaction product formed by the binding of cysteine to the 5-position of CQA can also be expected. No reaction products with dimers of CQA or caffeic acid with cysteine have been detected up to now. The mechanism would differ with the amino acid involved, because when they react with primary amino groups, phenolic compounds are supposed to dimerise prior to reacting with the amino acid (Namiki et al., 2001). This mechanism, in which a cyclization step occurs after binding of the amino acid, is shown in **Figure 13**.

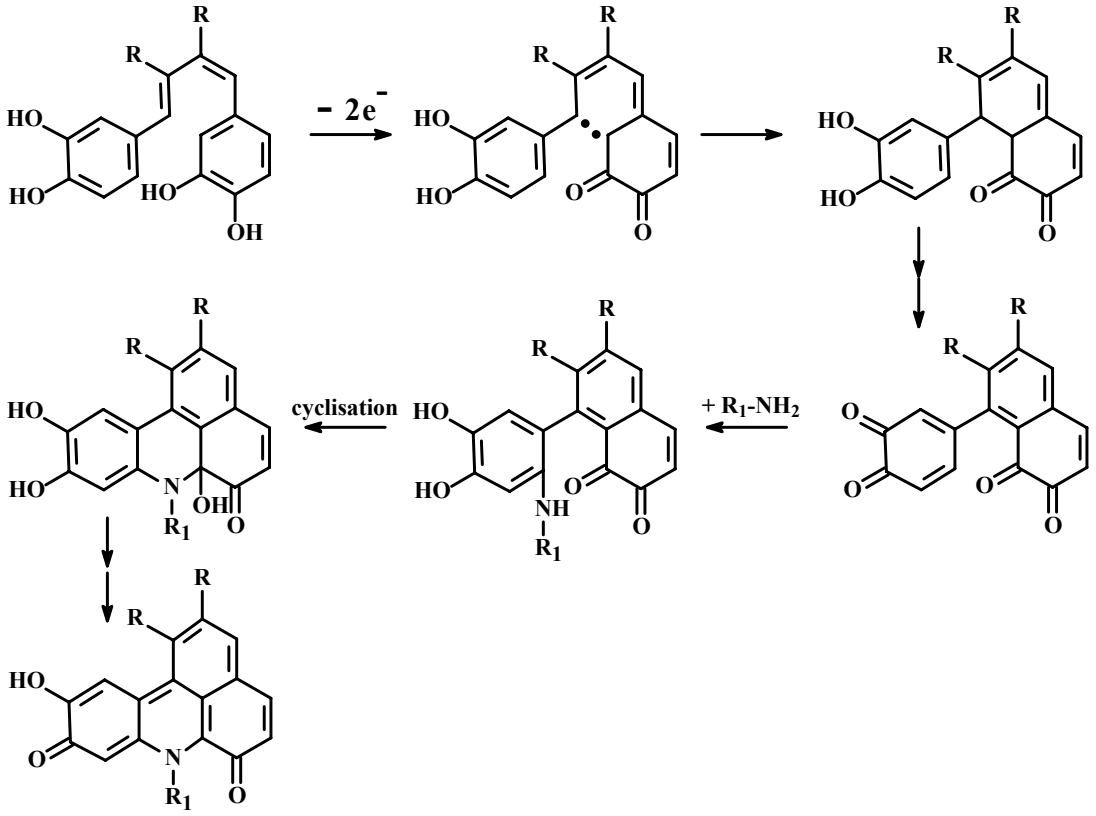


Figure 13. Proposed mechanism of reaction of dimerised caffeic acid ester (R = $COO-CH_2-CH_3$) with an amino compound (R_1-NH_2) (Namiki et al., 2001)

Since various structures of CQA dimers may exist, several reaction products with amino acids can be expected. Furthermore, which amino acid side-chains can react and how the structure of the reaction products formed looks like, either with a monomer or a dimer of CQA, remains to be clarified.

3.2.2 Covalent interactions with proanthocyanidins at acidic pH

As mentioned previously, proanthocyanidins can be degraded at low pH usually only upon heating, into anthocyanidins (Beart et al., 1985;Haslam, 1989;Porter et al., 1986;Swain and Hillis, 1959) (**Figure 14**).

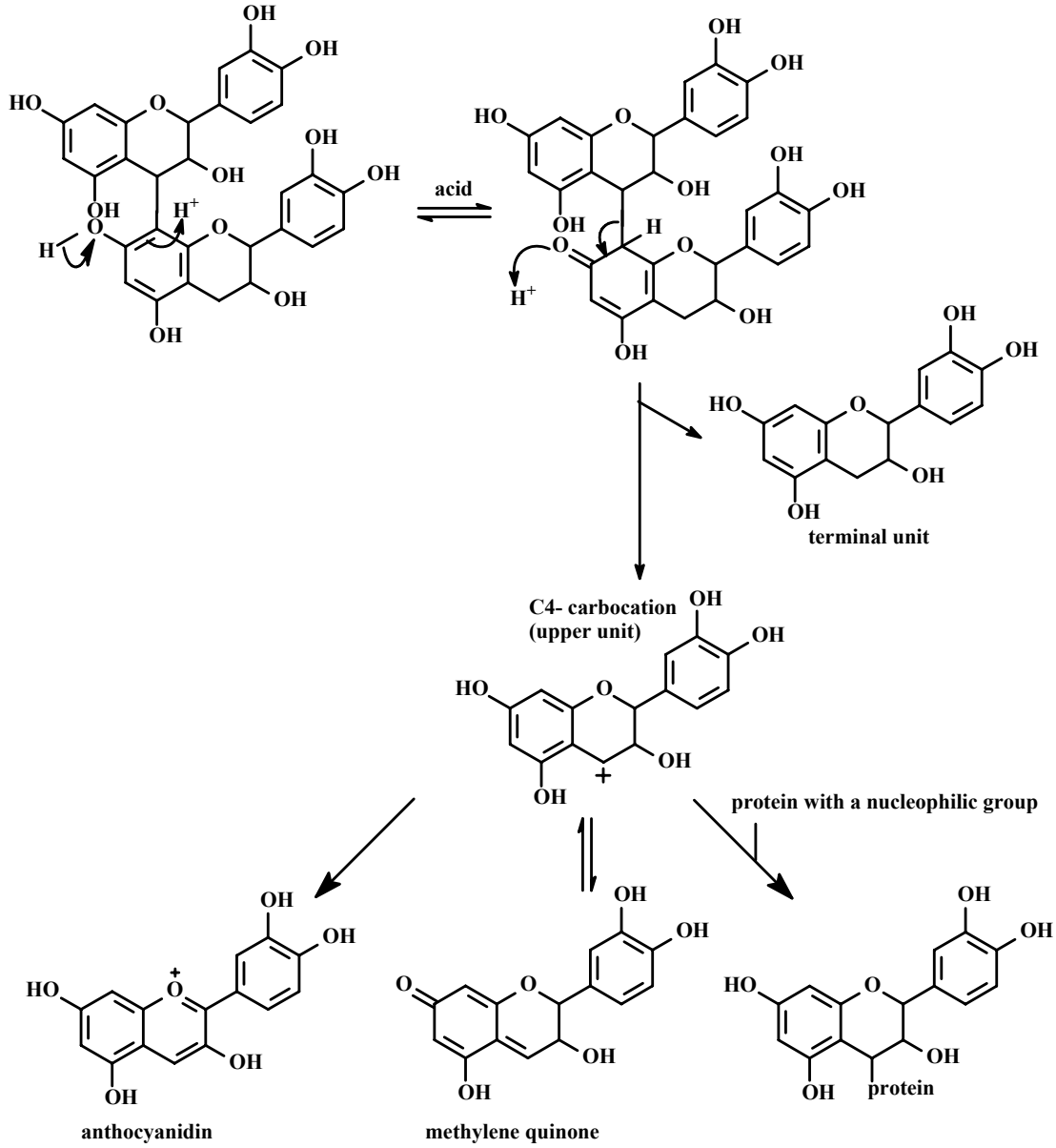


Figure 14: Acidic degradation of proanthocyanidins and reaction with nucleophilic groups (adapted from Beart et al., 1985;Haslam, 1989)

During depolymerization, the molecule of proanthocyanidin is cleaved into a terminal unit and a reactive carbocation (**Figure 13**). The carbocation is converted into an anthocyanidin or a quinone molecule or can react with any nucleophilic group (Guyot et al., 2001), among which those located on proteins (**Figure 14**). For example, the covalent binding of proanthocyanidins to proteins due to their depolymerization at low pH has been reported to occur between procyanidins and the thiol group of cysteamine (Selga et al., 2004).

3.2.3 Overview of the interactions

Figure 15 presents an overview of the interactions between phenolic compounds themselves and the interactions between phenolic compounds and proteins. As mentioned previously, non-covalent interactions are reversible, and covalent interactions are supposed to be, in most cases, irreversible.

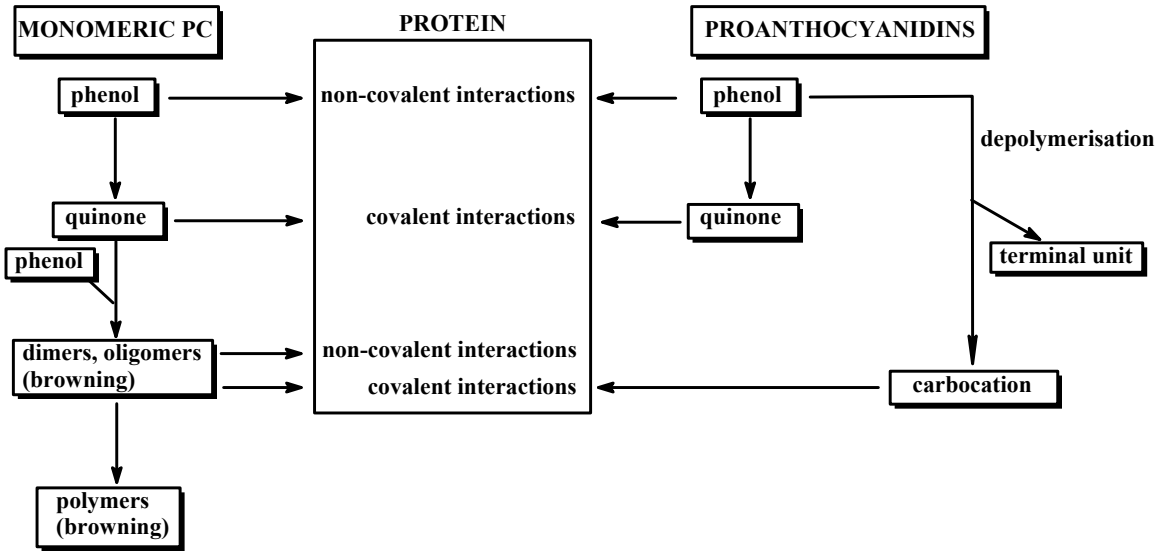


Figure 15. Overview of the phenolic compound (PC)- protein interactions

4. METHODS FOR STUDYING PHENOLIC COMPOUND–PROTEIN INTERACTIONS

4.1 Non-covalent interactions

As presented in **Table 1**, non-covalent interactions between phenolic compounds and proteins can be studied on the basis of different principles: separation according to molecular size (MW), charge, and by measuring spectroscopic properties and binding energy.

Table 1. Some methods for phenolic compound - protein studies and their limitations.

principle	method	sensitivity; reproducibility	limitations
MW separation	ED*	low reproducibility	binding to membrane; time-consuming
	Ultrafiltration	good	binding to membrane (but lower than with ED)
	HD-GF**	good	aspecific binding to column material; high amount of phenolic compounds consumed
	Precipitation	good	soluble phenolic compounds - protein complexes not measured
Charge separation	CE***	good	
Spectroscopy	Fluorescence	low sensitivity"*	based on environment of only some amino acids
	SPR*"	good	long set-up of parameters
Energy transfert	ITC*""	good	not suitable for very weak or very strong interactions

**ED = equilibrium dialysis; **HD-GF = method of Hummel and Dreyer used with gel filtration; ***CE = capillary electrophoresis;*" SPR = surface plasmon resonance;.*""ITC = isothermal titration calorimetry; ''*Fluorescence has an excellent sensitivity, but not for weak interactions.*

Complexes between phenolic compounds and proteins can be separated from free reactants based on differences in molecular size. For example, equilibrium dialysis (ED) and ultrafiltration are used to be able to measure the free ligand concentration, i.e. the free phenolic compound concentration. Using ultrafiltration, special care should be taken to avoid modification of the equilibrium between free and bound compounds: the volume, which goes through the membrane, and which is used to determine free ligand concentration should be small compared to the total volume, and the pressure on the membrane should be low. During ultrafiltration and ED, phenolic compounds can bind to the membrane used. Because of the relatively large membrane surface and the long exposure time, membrane-binding often leads to a lack of reproducibility. Aspecific binding to the column material can also occur in the case of gel filtration experiments. Gel filtration can be performed using the method of Hummel and Dreyer (Hummel and Dreyer, 1962). This method consists of injecting a protein solution into a continuous flow of free ligand, which maintains the equilibrium between bound ligand and free ligand (Busch et al., 1997;Hummel and Dreyer, 1962). Another technique based on molecular weight separation consists of measuring protein precipitation. However, it is an indirect method and because it does not take into account soluble phenolic compound-protein complexes, it cannot be used to measure binding constants.

Instead of molecular size separation, complexes and free reactants can also be separated based on differences in electrophoretic mobility, by capillary electrophoresis (CE). Several CE methods exist. In frontal analysis, a large plug of a phenolic compound-protein mixture can be

injected without modifying the equilibrium between the free and bound ligand during the migration (Busch et al., 1997). An additional advantage is that CE uses only low volumes of phenolic compound and proteins.

The formation of complexes can also be investigated by spectroscopy, e.g. diffraction of light, but it is usually an indirect measurement. Fluorescence spectroscopy e.g. only measures the changes in the environment of certain amino acid residues. Furthermore, fluorescence is not sufficiently sensitive to measure binding constants of very weak interactions. Another method, surface plasmon resonance, relies on measuring the amount of ligand bound to proteins via the diffraction of light at noble metal interfaces. This method, however, may require a lot of time to optimise the coupling. Nevertheless, an advantage of this method is that real-time measurements are performed. This is also the case for isothermal titration calorimetry (ITC). This technique is used to determine the affinity and the number of binding sites by measuring the enthalpy of transfer released or absorbed by the interactions. These changes are measured during successive additions of phenolic compound into a protein solution. ITC is a sensitive method, but conclusions are not easily drawn in situations of very weak interactions or very strong interactions.

4.2 Covalent interactions

Covalent interactions between phenolic compounds and proteins can be studied on molecular size or weight measurements using gel filtration chromatography, gel and capillary electrophoresis or mass spectrometry. Mass spectrometry permits to measure the exact molecular mass of molecules, whereas capillary electrophoresis, and to an even more pronounced way, gel electrophoresis and gel filtration chromatography, are less precise techniques. The structure of the covalent adducts can be determined by nuclear magnetic resonance (NMR), but the purity of the sample is crucial and extensive purification is needed when several compounds are formed upon modification.

5. AIM

Globular proteins are important proteins in foods and food ingredients. However, whereas interactions of phenolic compounds with proline-rich proteins, especially saliva PRP, have been extensively studied, interactions of phenolic compounds with globular proteins have not been studied in detail. In order to be able to control the isolation and the functional properties of proteins important in food industry, this study was aimed at characterising the covalent and

non-covalent interactions between monomeric and multimeric phenolic compounds and globular proteins, and their effects on the physico-chemical and functional properties of proteins.

6. PROTEINS STUDIED

Three globular proteins were chosen as model proteins in this study: bovine α-lactalbumin, chicken egg white lysozyme and bovine serum albumin (BSA), of which the amino acid compositions are presented in **Table 2**. α-Lactalbumin (14.2 kDa) and lysozyme (14.3 kDa) have a strongly similar sequence and structure (Sugai and Ikeguchi, 1994). Both proteins are composed of a α-helical domain and a β-sheet, and possess four disulfide bridges (Permyakov and Berliner, 2000;Sugai and Ikeguchi, 1994). In contrast to lysozyme, α-lactalbumin binds calcium, which increases its stability towards heat denaturation (Nieuwenhuizen et al., 2003). The proteins differ also in their isoelectric point (pI): α-lactalbumin and lysozyme possess a pI of 4.2-4.5 and 10.5-11.3, respectively. BSA possesses an isoelectric point close to that of α-lactalbumin (pI= 4.7-4.9), but has a higher molecular weight (66.4 kDa). The structure of BSA is roughly spherical and is characterized by a relatively high proportion of charged amino acids, and by nine double loops formed by 17 disulphides bonds (Carter and Ho, 1994). Because the optimum pH to form non-covalent complexes between oligomeric procyanidins and globular proteins is reported to be 0-3 pH units below the isoelectric point of globular proteins (De Freitas and Mateus, 2001b;Hagerman et al., 1998;Naczk et al., 1996), lysozyme can be expected to interact less strongly with phenolic compounds at pHs lower than 5.0 than α-lactalbumin and BSA. Due to its larger molecular size, BSA is expected to bind, on a molar basis, more phenolic compounds than α-lactalbumin and lysozyme. BSA also differs from α-lactalbumin and lysozyme with respect to hydrophobicity. The hydrophobicity value of BSA is much higher than that of α-lactalbumin, which in turn is higher than that of lysozyme (Li-Chan, 1990)(**Table 2**). It can thus be expected that hydrophobic interactions play a stronger role in phenolic compound-BSA interactions than in the interactions between phenolic compounds and α-lactalbumin or lysozyme.

Table 2. Amino acid (aa) composition of α-lactalbumin, lysozyme and BSA (http://www.expasy.org/sprot/sprot-top.html) and hydrophobicity measured using anilino-naphtalene sulfonic acid (ANS So) and cis-parinaric acid (CPA So)(Li-Chan, 1990). So = initial slope of relative fluorescence intensity versus protein concentration. The percentages are expressed as the number of each type of amino acids per one hundred amino acids.

	α-lactalbumin		lysozyme		BSA	
	number of aa	%	number of aa	%	number of aa	%
Ala (A)	3	2.4	12	9.3	47	8.1
Arg (R)	1	0.8	11	8.5	23	3.9
Asn (N)	8	6.5	14	10.9	14	2.4
Asp (D)	13	10.6	7	5.4	40	6.9
Cys (C)	8	6.5	8	6.2	35	6.0
Gln (Q)	6	4.9	3	2.3	20	3.4
Glu (E)	7	5.7	2	1.6	59	10.1
Gly (G)	6	4.9	12	9.3	16	2.7
His (H)	3	2.4	1	0.8	17	2.9
Ile (I)	8	6.5	6	4.7	14	2.4
Leu (L)	13	10.6	8	6.2	61	10.5
Lys (K)	12	9.8	6	4.7	59	10.1
Met (M)	1	0.8	2	1.6	4	0.7
Phe (F)	4	3.3	3	2.3	27	4.6
Pro (P)	2	1.6	2	1.6	28	4.8
Ser (S)	7	5.7	10	7.8	28	4.8
Thr (T)	7	5.7	7	5.4	33	5.7
Trp (W)	4	3.3	6	4.7	2	0.3
Tyr (Y)	4	3.3	3	2.3	20	3.4
Val (V)	6	4.9	6	4.7	36	6.2
ANS So		90		1		1600
CPA So		280		15		2750

7. OUTLINE OF THIS THESIS

The non-covalent interactions between proteins and CQA and their effects on protein solubility and heat denaturation behaviour are investigated, as presented in **chapter 2**. In **chapter 3**, the effects of covalent interactions with CQA quinones on protein mass and solubility are described. Covalent interactions at pH values close to neutral pH are compared to results obtained with modifications induced at alkaline pH. In **chapter 4**, emphasis is put on investigating which amino acid side-chains are able to react with CQA quinones. Structures of the formed covalent adducts are discussed. The possible reactions with amino acids are discussed in relation with the results that are observed with proteins.

Chapter 5 describes the interactions between proteins and oligomeric procyanidins under various environmental conditions and their effects on protein solubility and foam properties.

Finally, **chapter 6** presents a general overview of the main results, which are discussed and put in a broader perspective.

LITERATURE CITED

Amitai, G.; Adani, R.; Sod-Moriah, G.; Rabinovitz, I.; Vincze, A.; Leader, H.; Chefetz, B.; Leibovitz-Persky, L.; Friesem, D.; Hadar, Y. Oxidative biodegradation of phosphorothiolates by fungal laccase. *FEBS Lett.* **1998**, *438*, 195-200.

Antolovich, M.; Bedgood, D. R. J.; Bishop, A. G.; Jardine, D.; Prenzler, P. D.; Robards, K. LC-MS investigation of oxidation products of phenolic antioxidants. *J. Agric. Food Chem.* **2004**, *52*, 962-971.

Arts, I. C. W.; Hollman, P. C. H.; Kromhout, D. Chocolate as a source of tea flavonoids. *The Lancet* **1999**, *354*, 488.

Artz, W. E.; Bishop, P. D.; Dunker, A. K.; Schanus, E. G.; Swanson, B. G. Interaction of synthetic proanthocyanidin dimer and trimer with bovine serum albumin and purified bean globulin fraction G-1. *J. Agric. Food Chem.* **1987**, *35*, 417-421.

Bakowska, A.; Kucharska, A. Z.; Oszmianski, J. The effects of heating, UV irradiation, and storage on stability on the anthocyanin-polyphenol copigment complex. *Food Chem.* **2003**, *81*, 349-355.

Baxter, N. J.; Lilley, T. H.; Haslam, E.; Williamson, M. P. Multiple interactions between polyphenols and a salivary proline-rich protein repeat result in complexation and precipitation. *Biochemistry* **1997**, *36*, 5566-5577.

Beart, J. E.; Lilley, T. H.; Haslam, E. Polyphenol interactions. Part 2. Covalent binding of procyanidins to proteins during acid-catalysed decomposition; observations on some polymeric proanthocyanidins. *J. Chem. Soc., Perkin Trans. 2* **1985**, 1439-1443.

Bernillon, personal communication, **2005.**

Bernillon, S.; Renard, M. G. C.; Guyot, S. An LC/MS approach to study oxidation products of polyphenolic compounds in cider apple juice. *XXII International Conference on Polyphenols, Helsinki, Finland*; **2004**, Hoikkala, A.; Soidinsalo, O., Ed.; Gummerus Printing, p 557-558.

Beveridge, T.; Harrison, J. E.; Veto, L.; Pallares, E. N. Detection of filter media derived haze in apple juice concentrate. *Food Res. Int.* **1996**, *29*, 577-583.

Bianco, A.; Chiaccho, U.; Rescifina, A.; Romeo, G.; Uccella, N. Biomimetic supramolecular biophenol-carbohydrate and biophenol-protein models by NMR experiments. *J. Agric. Food Chem.* **1997**, *45*, 4281-4285.

Busch, M. H. A.; Kraak, J. C.; Poppe, H. Principles and limitations of methods available for the determination of binding constants with affinity capillary electrophoresis. *J. Chromatogr. A* **1997**, *777*, 329-353.

Carter, D. C.; Ho, J. X. Structure of serum albumin. *Adv. Protein Chem.* **1994**, *45*, 153-203.

Charlton, A. J.; Baxter, N. J.; Lokman, M. K.; Moir, A. J. G.; Haslam, E.; Davies, A. P.; Williamson, M. P. Polyphenol/peptide binding and precipitation. *J. Agric. Food Chem.* **2002**, *50*, 1593-1601.

Cheynier, V.; Osse, C.; Rigaud, J. Oxidation of grape juice phenolic compounds in model solutions. *J. Food Sci.* **1988**, *53*, 1729-1732.

Cilliers, J. J. L.; Singleton, V. L. Nonenzymic autoxidative phenolic browning reactions in a caffeic acid model system. *J. Agric. Food Chem.* **1989**, *37*, 890-896.

Cilliers, J. J. L.; Singleton, V. L. Caffeic acid autoxidation and the effects of thiols. *J. Agric. Food Chem.* **1990**, *38*, 1789-1796.

Cilliers, J. J. L.; Singleton, V. L. Characterization of the products of nonenzymic autoxidative phenolic reactions in a caffeic acid model system. *J. Agric. Food Chem.* **1991**, *39*, 1298-1303.

Clifford, M. N. Review. Chlorogenic acids and other cinnamates - nature, occurrence and dietary burden. *J. Sci. Food Agric.* **1999**, *79*, 362-372.

Clifford, M. N. Review. Chlorogenic acids and other cinnamates - nature, occurrence, dietary burden, absorption and metabolism. *J. Sci. Food Agric.* **2000**, *80*, 1033-1043.

De Freitas, V.; Mateus, N. Nephelometric study of salivary protein-tannin aggregates. *J. Sci. Food Agric.* **2001a**, *82*, 113-119.

De Freitas, V.; Mateus, N. Structural features of procyanidin interactions with salivary proteins. *J. Agric. Food Chem.* **2001b**, *49*, 940-945.

Felton, G. W.; Broadway, R. M.; Duffey, S. S. Inactivation of protease inhibitor activity by plant-derived quinones: complications for host-plant resistance against noctuid herbivores. *J. Insect Physiol.* **1989**, *35*, 981-990.

Fenoll, L. G.; Peñalver, M. J.; Rodríguez-López, J. N.; Varón, R.; García-Cánovas, F.; Tudela, J. Tyrosinase kinetics: discrimination between two models to explain the oxidation mechanism of monophenol and diphenol substrates. *Int. J. Biochem. Cell Biol.* **2004**, *36*, 235-246.

Fenoll, L. G.; Rodríguez-López, J. N.; García-Sevilla, F.; García-Ruiz, P. A.; Varón, R.; García-Cánovas, F.; Tudela, J. Analysis and interpretation of the action mechanism of mushroom tyrosinase on monophenols and diphenols generating highly unstable *o*-quinones. *Biochim. Biophys. Acta* **2001**, *1548*, 1-22.

Fenoll, L. G.; Rodríguez-López, J. N.; Varón, R.; García-Ruiz, P. A.; García-Cánovas, F.; Tudela, J. Kinetic characterisation of the reaction mechanism of mushroom tyrosinase on tyramine/dopamine and L-tyrosine methy esther/L-dopa methyl esther. *Int. J. Biochem. Cell Biol.* **2002**, *34*, 1594-1607.

Ferreira, R. B.; Piçarra-Pereira, M. A.; Monteiro, S.; Loureiro, V. B.; Teixeira, A. R. The wine proteins. *Trends in Food Science & Technology* **2002**, *12*, 230-239.

Frazier, R. A.; Papadopoulou, A.; Mueller-Harvey, I.; Kissoon, D.; Green, R. J. Probing protein-tannin interactions by isothermal titration microcalorimetry. *J. Agric. Food Chem.* **2003**, *51*, 5189-5195.

Friedman, M. Chemistry, biochemistry, and dietary role of potato polyphenols. A review. *J. Agric. Food Chem.* **1997**, *45*, 1523-1540.

Friedman, M.; Jürgens, H. S. Effect of pH on the stability of plant phenolic compounds. *J. Agric. Food Chem.* **2000**, *48*, 2101-2110.

Fulcrand, H.; Cheminat, A.; Brouillard, R.; Cheynier, V. Characterization of compounds obtained by chemical oxidation of caffeic acid in acidic conditions. *Phytochemistry* **1994**, *35*, 499-505.

García, P.; Concepción, R.; Brenes, M.; Garrido, A. Effect of metal cations on the chemical oxidation of olive *o*-diphenols in model systems. *J. Agric. Food Chem.* **1996**, *44*, 2101-2105.

Gonzalez-Perez, S.; Merck, K. B.; Vereijken, J. M.; Van Koningsveld, G. A.; Gruppen, H.; Voragen, A. G. J. Isolation and characterization of undenatured chlorogenic acid free sunflower (*Helianthus annuus*) proteins. *J. Agric. Food Chem.* **2002**, *50*, 1713-1719.

Guyot, S.; Marnet, N.; Sanoner, P.; Drilleau, J. F. Direct thiolysis on crude apple materials for high-performance liquid chromatography characterization and quantification of polyphenols in cider apple tissues and juices. *Methods Enzymol.* **2001**, *335*, 57-71.

Guyot, S.; Vercauteren, J.; Cheynier, V. Structural determination of colourless and yellow dimers resulting from (+)-catechin coupling catalysed by grape polyphenoloxidase. *Phytochemistry* **1996**, *42*, 1279-1288.

Hagerman, A. E.; Rice, M. E.; Ritchard, N. T. Mechanisms of protein precipitation for two tannins, pentagalloyl glucose and epicatechin$_{16}$ (4-->8) catechin (procyanidin). *J. Agric. Food Chem.* **1998**, *46*, 2590-2595.

Handique, J. G.; Baruah, J. B. Polyphenolic compounds: an overview. *React. Funct. Polym.* **2002**, *52*, 163-188.

Haribal, M.; Feeny, P.; Lester, C. C. A caffeoylcyclohexane-1-carboxylic acid derivative from *Asimina Triloba*. *Phytochemistry* **1998**, *49*, 103-108.

Harrison, C. R.; Hodge, P. Polymer-supported periodate and iodate as oxidizing agents. *J. Chem. Soc. [Perkin. 1]*. **1982**, 509-511.

Haslam, E. *Plant polyphenols: vegetable tannins revisited*; Phillipson, J.D.; Ayres, D.C.; Baxter, H., Eds.; Cambridge University Press: Cambridge, 1989, 230 p.

Haslam, E. Natural polyphenols (vegetable tannins) as drugs: possible modes of action. *J. Nat. Prod.* **1996**, *59*, 205-215.

Hemingway, R. W. *Chemistry and significance of condensed tannins*; Hemingway, R.W.; Karchesy, J., Eds.; Plenum press: New York, 1989, 83-107.

Hummel, J. P.; Dreyer, W. J. Measurement of protein-binding phenomena by gel filtration. *Biochim. Biophys. Acta* **1962**, *63*, 530-532.

Hurrell, R. F.; Finot, P. A.; Cuq, J. L. Protein-polyphenol reactions. 1. Nutritional and metabolic consequences of the reaction between oxidized caffeic acid and the lysine residues of casein. *Br. J. Nutr.* **1982**, *47*, 191-211.

Kawabata, J.; Okamoto, Y.; Kodama, A.; Makimoto, T.; Kasai, T. Oxidative dimers produced from protocatechuic and gallic esters in the DPPH radical scavenging reaction. *J. Agric. Food Chem.* **2002**, *50*, 5468-5471.

Kerwin, J. L.; Turecek, F.; Xu, R.; Kramer, K. J.; Hopkins, T. L.; Gatlin, C. L.; Yates III, J. R. Mass spectrometric analysis of catechol-histidine adducts from insect cuticle. *Anal. Biochem.* **1999**, *268*, 229-237.

Kramer, K. J.; Kanost, M. R.; Hopkins, T. L.; Jiang, H.; Zhu, Y. C. Oxidative conjugation of catechols with proteins in insect skeletal systems. *Tetrahedron* **2001**, *57*, 385-392.

Kroll, J.; Rawel, H. M.; Rohn, S. Reactions of plant phenolics with food proteins and enzymes under special consideration of covalent bonds. *Food Sci. Technol. Res.* **2003**, *9*, 205-218.

Lakenbrink, C.; Lapcynski, S.; Maiwald, B.; Engelhardt, U. H. Flavonoids and other polyphenols in consumer brews of tea and other caffeinated beverages. *J. Agric. Food Chem.* **2000**, *48*, 2848-2852.

Li-Chan, E. Hydrophobicity in food protein systems. In *Encyclopedia of food science and technology*; Y. H. Hui, Ed.; Wiley-Interscience: New York, 1990, 1429-1439.

Matheis, G.; Whitaker, J. R. Modification of proteins by polyphenol oxidase and peroxidase and their products. *J. Food Biochem.* **1984**, *8*, 137-162.

Mathew, A. G.; Lakshminarayana, S. Polyphenols of immature sapota fruit. *Phytochemistry* **1969**, *8*, 507-509.

Mayer, A. M. Polyphenol oxidases in plants - recent progress. *Phytochemistry* **1987**, *26*, 11-20.

Mayer, A. M.; Harel, E. Polyphenol oxidases in plants. *Phytochemistry* **1979**, *18*, 193-215.

Mayer, A. M.; Staples, R. C. Laccase: new functions for an old enzyme. *Phytochemistry* **2002**, *60*, 551-565.

McCord, J. D.; Kilara, A. Control of enzymatic browning in processed mushrooms (*Agaricus bisporus*). *J. Food Sci.* **1983**, *48*, 1479-1483.

Montavon, P.; Mauron, A.-F.; Duruz, E. Changes in green coffee protein profiles during roasting. *J. Agric. Food Chem.* **2003**, *51*, 2335-2343.

Naczk, M.; Oickle, D.; Pink, D.; Shahidi, F. Protein precipitating capacity of crude canola tannins: effect of pH, tannin, and protein concentrations. *J. Agric. Food Chem.* **1996**, *44*, 2144-2148.

Namiki, M.; Yabuta, G.; Koizumi, Y.; Yano, M. Development of free radical products during the greening reaction of caffeic acid esters (or chlorogenic acid) and a primary amino compound. *Biosci. Biotechnol. Biochem.* **2001**, *65*, 2131-2136.

Negishi, O.; Ozawa, T. Inhibition of enzymatic browning and protection of sulfhydryl enzymes by thiol compounds. *Phytochemistry* **2000**, *54*, 481-487.

Nieuwenhuizen, W. F.; Dekker, H. K.; de Koning, L. J.; Gröneveld, T.; de Koster, C. G.; de Jong, G. A. H. Modification of glutamine and lysine residues in holo and apo α-lactalbumin with microbial transglutaminase. *J. Agric. Food Chem.* **2003**, *51*, 7132-7139.

O'Connell, J. E.; Fox, P. F. Significance and applications of phenolic compounds in the production and quality of milk and dairy products: a review. *Int. Dairy. J.* **2001**, *11*, 103-120.

Osuga, D.; van der Schaaf, A.; Whitaker, J. R. *Protein structure-function relationships in foods. 4. Control of polyphenol oxidase activity using a catalytic mechanism*; Yada, R.Y; Jackman, R.L.; Smith, J.L., Eds. Chapman & Hall, 1994, 62-88.

Oszmianski, J.; Lee, C. Y. Enzymatic oxidative reaction of catechin and chlorogenic acid in a model system. *J. Agric. Food Chem.* **1990**, *38*, 1202-1204.

Parr, A. J.; Bolwell, G. P. Phenols in the plant and in man. The potential for possible nutritional enhancement of the diet by modifying the phenols content or profile. *J. Sci. Food Agric.* **2000**, *80*, 985-1012.

Peñalver, M. J.; Hiner, A. N. P.; Rodríguez-López, J. N.; García-Cánovas, F.; Tudela, J. Mechanistic implications of variable stoichiometries of oxygen consumption during tyrosinase catalyzed oxidation of monophenols and *o*-diphenols. *Biochim. Biophys. Acta* **2002**, *1597*, 140-148.

Permyakov, E. A.; Berliner, L. J. α-Lactalbumin: structure and function. *FEBS Lett.* **2000**, *473*, 269-274.

Pierpoint, W. S. The enzymic oxidation of chlorogenic acid and some reactions of the quinone produced. *Biochem. J.* **1966**, *98*, 567-580.

Pierpoint, W. S. o-Quinones formed in plant extracts. Their reaction with bovine serum albumin. *Biochem. J.* **1969a**, *112*, 619-629.

Pierpoint, W. S. *o*-Quinones formed in plant extracts. Their reactions with amino acids and peptides. *Biochem. J.* **1969b**, *112*, 609-616.

Pierpoint, W. S. A class of blue quinone-protein coupling products: the allagochromes? *Phytochemistry* **1982**, *21*, 91-95.

Porter, L. J.; Hrstich, L. N.; Chan, B. G. The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. *Phytochemistry* **1986**, *25*, 223-230.

Rawel, H. M.; Kroll, J.; Riese, B. Reactions of chlorogenic acid with lysozyme: physicochemical characterization and proteolytic digestion of the derivatives. *J. Food Sci.* **2000**, *65*, 1091-1098.

Rawel, H. M.; Rohn, S.; Kruse, H.-P.; Kroll, J. Structural changes induced in bovine serum albumin by covalent attachment of chlorogenic acid. *Food Chemistry* **2002**, *78*, 443-455.

Richard, F. C.; Goupy, P. M.; Nicolas, J. J.; Lacombe, J.-M.; Pavia, A. A. Cysteine as an inhibitor of enzymatic browning. 1. Isolation and characterization of addition compounds formed during oxidation of phenolics by apple polyphenol oxidase. *J. Agric. Food Chem.* **1991**, *39*, 841-847.

Richard-Forget, F. C.; Gauillard, F. A. Oxidation of chlorogenic acid, catechins, and 4-methylcatechol in model solutions by combinations of pear (*Pyrus communis* Cv. Williams) polyphenol oxidase and peroxidase; a possible involvement of peroxidase in enzymatic browning. *J. Agric. Food Chem.* **1997**, *45*, 2472-2476.

Richard-Forget, F. C.; Goupy, P. M.; Nicolas, J. J. Cysteine as an inhibitor of enzymatic browning. 2. Kinetic studies. *J. Agric. Food Chem.* **1992a**, *40*, 2108-2113.

Richard-Forget, F. C.; Rouet-Mayer, M.-A.; Goupy, P. M.; Philippon, J.; Nicolas, J. J. Oxidation of chlorogenic acid, catechins, and 4-methylcatechol in model solutions by apple polyphenol oxidase. *J. Agric. Food Chem.* **1992b**, *40*, 2114-2122.

Richelle, M.; Tavazzi, I.; offord, E. Comparison of the antioxidant activity of commonly consumed polyphenolic beverages (coffee, cocoa, and tea) prepared per cup serving. *J. Agric. Food Chem.* **2001**, *2001*, 3438-3442.

Robards, K.; Prenzler, P. D.; Tucker, G.; Swatsitang, P.; Glover, W. Phenolics compounds and their role in oxidative processes in fruits. *Food Chem.* **1999**, *66*, 401-436.

Robb, D. A.; Gutteridge, S. Polypeptide composition of two fungal tyrosinases. *Phytochemistry* **1981**, *20*, 1481-1485.

Rodríguez-López, J. N.; Fenoll, L. G.; Peñalver, M. J.; García-Ruiz, P. A.; Varón, R.; Martínez-Ortíz, F.; García-Cánovas, F.; Tudela, J. Tyrosinase action on monophenols: evidence for direct enzymatic release of *o*-diphenol. *Biochim. Biophys. Acta* **2001**, *1548*, 238-256.

Rompel, A.; Fischer, H.; Meiwes, D.; Buldt-Karentzopoulos, K.; Magrini, A.; Eicken, C.; Gerdemann, C.; Krebs, B. Substrate specificity of catechol oxidase from *Lycopus europaeus* and characterization of enzymic caffeic acid oxidation. *FEBS Lett.* **1999**, *445*, 103-110.

Sanoner, P. Les polyphénols de la pomme à cidre: Diversité variétale et oxydation, Doctorat de l'Université de Caen / Basse-Normandie, 2001.

Santos-Buelga, C.; Scalbert, A. Proanthocyanidins and tannin-like compounds - nature, occurence, dietary intake and effects on nutrition and health. *J. Sci. Food Agric.* **2000**, *80*, 1094-1117.

Selga, A.; Sort, X.; Bobet, R.; Torres, J. L. Efficient one pot extraction and depolymerization of grape (*Vitis vinifera*) pomace procyanidins for the preparation of antioxidant thio-conjugates. *J. Agric. Food Chem.* **2004**, *52*, 467-473.

Shahidi, F.; Naczk, M. *Food phenolics: sources, chemistry, effects, applications*; Technomic Publishing Company: Lancaster, USA, 1995, p. 331.

Siebert, K. J. Effect of protein-polyphenol interactions on beverage haze, stabilization, and analysis. *J. Agric. Food Chem.* **1999**, *47*, 353-362.

Strauss, G.; Gibson, S. M. Plant phenolics as cross-linkers of gelatin gels and gelatin-based coacervates for use as food ingredients. *Food Hydrocolloids* **2004**, *18*, 81-89.

Sugai, S.; Ikeguchi, M. Conformational comparison between α-lactalbumin and lysozyme. *Adv. Biophys.* **1994**, *30*, 37-84.

Swain, T.; Hillis, W. E. The phenolic constituents of *Prunus domestica*. I. The quantitative analysis of phenolic constituents. *J. Sci. Food Agric.* **1959**, *10*, 63-68.

Tazaki, H.; Taguchi, D.; Hayashida, T.; Nabeta, K. Stable isotope-labeling studies on the oxidative coupling of caffeic acid via *o*-quinone. *Biosci. Biotechnol. Biochem.* **2001**, *65*, 2613-2621.

Tulyathan, V.; Boulton, R. B.; Singleton, V. L. Oxygen uptake by gallic acid as a model for similar reactions in wines. *J. Agric. Food Chem.* **1989**, *37*, 844-849.

USDA database. Database for the proanthocyanidin content of selected foods. *htttp://www.nal.usda.gov/fnic/foodcomp* **2004**.

Van Koningsveld, G.; Gruppen, H.; de Jongh, H. J.; Wijngaards, G.; van Boekel, M. A. J. S.; Walstra, P.; Voragen, A. G. J. The solubility of potato proteins from industrial potato fruit juice as influenced by pH and various additives. *J. Sci. Food Agric.* **2002**, *82*, 134-142.

Vithayathil, P. J.; Satyanarayana Murthy, G. New reaction of *o*-benzoquinone at the thioether group of methionine. *Nature. New Biol.* **1972**, *236*, 101-103.

Wallerath, T.; Li, H.; Gödtel-Ambrust, U.; Schwarz, P. M.; Förstermann, U. A blend of polyphenolic compounds explains the stimulatory effect of red wine on human endothelial NO synthase. *Nitric Oxide* **2005**, *12*, 97-104.

Xu, R.; Huang, X.; Morgan, T. D.; Prakash, O.; Kramer, K. J.; Hawley, M. D. Characterization of products from the reactions of *N*-acetyldopamine quinone with *N*-acetylhistidine. *Arch. Biochem. Biophys.* **1996**, *329*, 56-64.

Yabuta, G.; Koizumi, Y.; Namiki, K.; Hida, M.; Namiki, M. Structure of green pigment formed by the reaction of caffeic acid esters (or chlorogenic acid) with a primary amino compound. *Biosci. Biotechnol. Biochem.* **2001**, *65*, 2121-2130.

Zhu, J. H.; Gray, D. G. Photoyellowing of lignin-rich paper: interaction of excited states with selected additives. *J. Photochem. Photobiol., A* **1995**, *87*, 267-273.

Chapter 2

Effects of non-covalent interactions with caffeoylquinic acid (chlorogenic acid) on the heat denaturation and solubility of globular proteins

Chapter 2

Effects of non-covalent interactions with caffeoylquinic acid (chlorogenic acid) on the heat denaturation and solubility of globular proteins*

The non-covalent interactions between the monomeric phenolic compound CQA (chlorogenic acid) and bovine serum albumin (BSA), lysozyme and α-lactalbumin were characterised and their effect on protein properties was examined. All three proteins had a low affinity for CQA and the interactions observed seemed to show a negative cooperativity. CQA-BSA binding decreased with increasing temperature, whereas pH (pH 3.0 vs. pH 7.0) and ionic strength had no pronounced effect. At high CQA/protein molar ratios, the denaturation enthalpy and temperature of BSA both increased, presumably due to the formation of covalent bonds at high temperatures. The presence of CQA had no effect on the solubility of BSA and α-lactalbumin as a function of pH, whereas it decreased lysozyme solubility at alkaline pH due to covalent interactions. These results indicate that the non-covalent interactions with CQA do not have pronounced effects on the functional properties of globular proteins in food systems.

KEYWORDS: phenolic compounds; BSA; lysozyme; α-lactalbumin; pH; temperature; ionic strength

*Based on:

Stéphanie V.E. Prigent, Harry Gruppen, Antonie J.W.G. Visser, Gerrit A. van Koningsveld, Govardus A.H. de Jong, and Alfons G.J. Voragen. Effects of non-covalent interactions with 5-O-caffeoylquinic acid (chlorogenic acid) on the heat denaturation and solubility of globular proteins. J. Agric. Food. Chem. **2003,** 51, 5088-5095

1. INTRODUCTION

Phenolic compounds are secondary plant metabolites with a broad range of chemical structures. Phenolics that are able to precipitate alkaloids, gelatin and globular proteins from solutions are usually called tannins, as defined by Bate-Smith and Swain in 1962 (Bate-Smith and Swain, 1962). The interaction of phenolics with proteins is also the main cause of undesirable haze in beer, wine and clear fruit juices (Siebert, 1999). In potato juice from potato starch industry, phenolic compounds are considered to be responsible for a decrease in protein solubility during protein recovery (Van Koningsveld et al., 2002). Phenolics can also have positive aspects by improving foam formation and stability (Sarker et al., 1995). The addition of phenolics from green tea results in an increased heat stability of milk, underlining the possible use of phenolics as food additives (O'Connell and Fox, 1999). Controlling the interactions between proteins and phenolics would therefore provide a tool to improve the functionality of plant proteins in foods. This is, however, only possible if the mechanisms underlying these interactions are known.

Plant phenolics may interact covalently or non-covalently with proteins. Both ways can lead to precipitation of proteins, either via multi-site interactions (several phenolics bound to one protein molecule), or multi-dentate interactions (one phenolic bound to several protein sites or protein molecules). Which type of interaction occurs will depend on the molar ratio phenolic/protein (Haslam, 1989).

The non-covalent interactions between phenolic compounds and proteins have been suggested to be created by hydrophobic association, which may subsequently be stabilised by hydrogen bonding (Haslam, 1989;Murray et al., 1994). However, whereas quite some research has been devoted to interactions between tannins and proline-rich proteins (Baxter et al., 1997;Siebert, 1999), the nature and extent of the non-covalent interactions between monomeric phenolics and globular proteins are still unclear. The interactions between tannins and proline-rich proteins may be due to π - bonded complexes formed by the overlapping of the rings of the phenolic and those of aromatic amino acid residues in proteins (Baxter et al., 1997). Proanthocyanidins are supposed to interact with bovine serum albumin (BSA) via hydrophobic interactions (Artz et al., 1987), whereas hydrogen bonding seems to be responsible for the interactions between 11S sunflower protein and 5-*O*-caffeoylquinic acid (Shamanthaka Sastry and Narasinga Rao, 1990), caffeic acid or quinic acid. In contrast to *p*-hydroxybenzoic acid, protocatechuic acid (3,4 dihydroxybenzoic acid) and caffeic acid have

been observed to interact with BSA, which has been suggested to reduce their antioxidant properties (Bartolomé et al., 2000).

The monomeric phenolic compound, 5`-*O*-caffeoylquinic acid (CQA / chlorogenic acid), is the most common individual compound of the cinnamic acid family (Clifford, 2000), and often represents the majority of the phenolic compounds in fruits (Robards et al., 1999). In this study, CQA was used as a model compound for cinnamic acid derivatives. The interactions of CQA with the globular proteins, BSA, α-lactalbumin, lysozyme, were studied. α-Lactalbumin has a chemical structure very similar to that of lysozyme, but has a lower isoelectric point and a lower structural stability (Privalov, 1979).

This paper presents a study on the interactions of CQA with globular proteins concerning binding constants, effects of environmental parameters (ionic strength, pH, temperature) on the interactions between CQA and proteins, and the effects of these interactions on protein properties (thermal denaturation and solubility) using a variety of techniques and methods.

2. MATERIAL AND METHODS

2.1 Materials

Bovine serum albumin (BSA, fractionated by cold alcohol precipitation and essentially fatty acid free), α-lactalbumin (type I), lysozyme (from chicken egg white) and 5'-caffeoylquinic acid (CQA; 1',3',4',5'-tetrahydroxycyclohexanecarboxylic acid 3-(3,4)-dihydroxypropenyldihydroxyphenyl-1-propenoate) were purchased from Sigma Chemical Company (St Louis, MO, USA) and used without further purification. The buffer used for gel filtration chromatography experiments and determination of the binding parameters at low ionic strength (equilibrium dialysis and technique of Hummel and Dreyer) was 0.020 M potassium phosphate buffer (pH 7.0; ionic strength (I) = 0.062 M) containing 0.02 M NaCl. All the other experiments were performed in 0.100 M potassium phosphate (pH 7.0; I = 0.184 M) to obtain a higher ionic strength, with the exception of solubility experiments for which a very low ionic strength was chosen (I= 0.017 M). Furthermore, a buffer of pH 3.0 (0.203 M potassium phosphate buffer) was used for studying the effect of low pH on the interactions, with a similar ionic strength to the 0.100 M potassium phosphate buffer of pH 7.0. The protein concentrations were similar for gel filtration and the techniques used to determine the binding parameters and the effects of pH and temperature (equilibrium dialysis, technique of Hummel and Dreyer, ultrafiltration). For isothermal titration calorimetry, differential scanning

calorimetry and protein solubility experiments, the protein concentrations were chosen to fit the sensitivities of the techniques.

2.2 Gel filtration chromatography

Samples of 25 µl of 1.2 % (w/v) BSA were incubated with 0.02 M potassium phosphate buffer (pH 7.0) containing 0.02 M NaCl, for five minutes or 24 hours, at room temperature, with or without 0.02 % (w/v) CQA, and were subsequently applied to a Superdex-200 gel filtration column (3.2 x 300 mm; flow rate: 0.1 ml / min, Amersham Pharmacia Biotech, Uppsala, Sweden) on a SMART system (Amersham Pharmacia Biotech, Uppsala, Sweden). Protein elution was detected at 280 nm, CQA elution at 324 nm.

2.3 Reversibility of CQA interactions

Samples of 0.01 % (w/v) BSA in 0.1 M potassium phosphate buffer (pH 7.0) containing 0.02% (w/w) of sodium azide were incubated during 48 hours with CQA at a ratio of 4 moles of CQA per mole of protein. Blanks without CQA were incubated during the same time. After incubation, all samples were dialysed for three hours at room temperature in Slide-A-Lyzer mini-dialysis units (Pierce Chemical Company, Rockford, IL, USA) with a molecular weight cut-off of 10 kDa against buffer. The removal of CQA was checked by measuring the absorbance of the eluate at 324 nm in a spectrophotometer. The experiment was performed in duplicate.

Samples of 0.1 % (w/v) lysozyme and α-lactalbumin were incubated, in 0.1 M potassium phosphate buffer (pH 7.0), during 15 minutes with CQA at a ratio of 300 moles of CQA per mole of protein. Blanks without CQA were incubated during the same time. After incubation, all samples were dialysed overnight in Slide-A-Lyzer mini-dialysis units (Pierce Chemical Company, Rockford, IL, USA) with a molecular weight cut-off of 10 kDa against distilled water at room temperature. Samples were further studied using MALDI-TOF MS analysis. The experiment was performed in duplicate.

2.4 MALDI-TOF MS analysis

Matrix assisted laser desorption/ionization - time of flight mass spectrometry (MALDI-TOF MS) analysis was performed following the drying droplet method using sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid, Sigma, St Louis, MO, USA) as a matrix dissolved to a concentration of 10 mg/ml in 50 % (v/v) acetonitrile containing 0.3 % (v/v) trifluoroacetic

acid (TFA). After external calibration using insulin (5.735 kDa), thioredoxin (11.674 kDa) and apo-myoglobin (16.953 kDa) (PerSeptive Biosystem, Framingham, MA, USA), 1 µl of the solutions was analysed on a Voyager^{TM} DE-RP MALDI-TOF MS (PerSeptive Biosystems, Framingham, MA, USA). Each sample was analysed in duplicate.

2.5 Equilibrium dialysis

Equilibrium dialysis experiments were performed at 25 ^{o}C during 16 hours in an apparatus from Dianorm GmbH with neutral cellulose dialysis membranes (molecular weight cut-off 5 kDa, Dianorm GmbH, Munich, Germany). One ml solutions of various concentrations of CQA, in 0.02 M potassium phosphate buffer (pH 7.0) containing 0.02 M NaCl, were dialysed against the same volume of 1.2 % (w/v) BSA in the same buffer. To determine the amount of CQA bound to the membrane, CQA was dialysed against buffer without BSA. After dialysis, the free CQA concentration (CQA_{free}) was measured at 324 nm in a spectrophotometer using a molar extinction coefficient of 18,500 M^{-1} cm^{-1} (Shamanthaka Sastry and Narasinga Rao, 1990). After correction for the CQA bound to the membrane, the number of the CQA molecules bound per molecule of protein (ν) was calculated. The binding constants, k_i, and the number of binding sites (n_i) were determined using a Scatchard plot representing $\nu/[CQA_{free}]$ as a function of ν (Scatchard, 1949). The curve was analysed with a graphic parameter fitting for two sets of binding sites (Weder et al., 1974). The curve was resolved into two straight lines, each line representing one set of binding sites, and their interceptions with the x and y axis representing n_i, and n_i x k_i, respectively. Each CQA-BSA ratio was studied ten times.

2.6 Hummel and Dreyer analysis

Binding experiments were performed on a ÄKTA purifier (Amersham Pharmacia Biotech, Uppsala, Sweden) according to the protocol of Hummel and Dreyer (Hummel and Dreyer, 1962) with a Superdex-30 PG gel filtration column (0.5 x 9.5 cm; flow: 0.4 ml / min) (Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated with the different running buffers containing different concentrations (0 - 1.7 mM) of CQA. Buffers used were 0.02 M potassium phosphate buffer (pH 7.0; ionic strength (I) = 0.062 M), containing 0.02 M NaCl, and 0.1 M potassium phosphate buffer (pH 7.0; I = 0.184 M). Samples applied were 25 µL of a solution containing 1.2 % (w/v) BSA and various CQA concentrations. Hummel-Dreyer

experiments were also performed with 1.5% (w/v) lysozyme in 0.02 M potassium phosphate buffer (pH 7.0) containing 0.02 M NaCl. The binding of CQA from the elution buffer to protein decreased the concentration of free CQA, creating a trough in the baseline of which the area was quantified, at 324 nm, 380 nm or 400 nm depending on the absorbance observed. The results from the Hummel and Dreyer method satisfied the criteria necessary to obtain correct binding parameters according to Busch and colleagues (Busch et al., 1997). The binding constants and number of binding sites were determined with graphic parameter fitting as described before. Four different protein-CQA ratio were performed for each CQA concentration in the elution buffer.

2.7 Isothermal titration calorimetry

The heat effect of successive additions of CQA on BSA was measured with a MCS isothermal titration calorimeter (MicroCal, Inc., Northampton, MA, USA) at 25 ^{o}C. Solutions of BSA and CQA in 0.1 M potassium phosphate buffer (pH 7.0) were degassed during 25 minutes under vacuum. The reference cell contained the degassed buffer. The sample cell contained 0.72% (w/v) BSA, while 0.89% (w/v) CQA was in a 250 μL syringe. The initial delay before the first injection was 600 seconds. Every 300 seconds, the syringe injected 10 μL of CQA in the BSA solution in a span of 13 seconds. The reference offset was 50 %. Samples with only BSA or CQA were used as blanks, and the data points of only CQA were subtracted from data points of BSA with CQA. The heat changes were analysed with the use of the Origin software (MicroCal Software, Inc., Northampton, MA, USA).

2.8 Differential scanning calorimetry

Samples used for differential scanning calorimetry (DSC) experiments were 0.05% (w/v) BSA, 0.1% (w/v) α-lactalbumin, 0.1% (w/v) lysozyme in 0.1 M potassium phosphate buffer (pH 7.0). After filtration over a 0.45 μm cellulose acetate filter (less than 5% of protein or CQA concentration lost), the proteins were incubated with various concentrations of CQA, degassed and injected in the sample cell of a VP-DSC calorimeter (MicroCal Inc., Northampton, MA, USA). The reference cell contained buffer with the same amount of CQA as the sample cell. The differential heat capacity was measured at a scanning rate of 60 ^{o}C per hour, from 25 ^{o}C to 70 ^{o}C, 80 ^{o}C or 90 ^{o}C for BSA, α-lactalbumin and lysozyme, respectively. Data were analysed with the MicroCal Origin software (MicroCal Inc., Northampton, MA,

USA). The phase transition temperature, T_m, was defined as the temperature at the maximum of the peak. Each experiment was repeated at least three times.

To investigate the presence of covalent modifications due to heating, samples of α-lactalbumin and lysozyme incubated with CQA at a ratio of 50 moles CQA per mole of protein were heated until 80 ^oC and 85 ^oC, respectively, at a scanning rate of 60 ^oC per hour. Subsequently, the samples were desalted by centrifugation in Microcon YM-10 centrifugation devices (Amicon, Millipore Corporation, Beverly, MA, USA), and then analysed using MALDI-TOF MS, as described previously. Blanks consisted of protein heated using DSC without CQA, and unheated protein.

2.9 Effect of temperature on CQA - protein interactions

To determine the effect of temperature on the CQA/BSA interactions, the amount of CQA bound to 1.2% (w/v) BSA was determined by the technique of Hummel and Dreyer (Hummel and Dreyer, 1962) at 5, 25 and 60^oC in 0.1 M potassium phosphate buffer (pH 7.0). The buffer and the Superdex-30 PG gel filtration column (0.5 x 9.5 cm; flow: 0.4 ml / min) (Amersham Pharmacia Biotech, Uppsala, Sweden) were thermostatted by a water bath. Four different protein-CQA ratio were performed for each CQA concentration in the elution buffer.

2.10 Effect of pH on CQA - protein interactions

Samples of 1.2 % (w/v) of BSA were incubated with CQA at different CQA/protein ratios in 0.1 M potassium phosphate buffer (pH 7.0), or in 0.2 M potassium phosphate buffer (pH 3.0). A small part of the unbound CQA was removed by ultrafiltration, (5 minutes, 16 x *g*, 25^oC) through membranes of 3 kDa molecular weight cut-off, with MPS micropartition devices (Amicon, Millipore Corporation, Beverly, MA, USA). After dilution with buffer, the CQA concentration of the filtrate was quantified at 324 nm in a spectrophotometer. Blanks used to correct for CQA bound to the filtration unit or to the membrane consisted of CQA without protein. Four replicates were performed per CQA-protein ratio.

2.11 Effect of protein denaturation on CQA - protein interactions

Samples of 2.4% (w/v) BSA and α-lactalbumin were denatured by DSC as described before, but without prior filtration, by heating for at least 30 minutes at 70 ^oC or 90 ^oC, respectively. Heated samples, and a calibration mixture (14.2 - 2,000 kDa) for molecular weight estimation, were applied on a Superdex-200 gel filtration column (30 x 0.35 cm; flow rate: 0.1 ml/min)

(Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated with 0.1 M potassium phosphate buffer (pH 7.0) in order to estimate the amounts and sizes of potentially present aggregated proteins. The eluate was monitored at 280 nm.

To quantify the amounts of CQA bound to heated proteins (0.13 % (w/v)), 10 moles of CQA were added per mole of protein. Subsequently, the unbound CQA was partly removed by ultrafiltration, as described before (four replicates).

2.12 Effect on protein solubility

The protein concentrations used were 0.1% (w/v) BSA, 0.1% (w/v) α-lactalbumin and 0.2% (w/v) lysozyme. The proteins were dissolved in 0.01 M potassium phosphate buffer (pH 7.0), with 100 moles of CQA per mole of protein. After filtration of the solutions, the samples were adjusted to various pH's with NaOH or HCl (1 M or 6 M). After two hours, the samples were centrifuged (15 min, 12,700 x *g*, 25^{o}C). The protein concentrations of the supernatants were measured at 595 nm in a spectrophotometer using the quantitative Coomassie Blue assay modified by Boyes et al. (Boyes et al., 1997). Blanks consisted of protein without CQA, and CQA without protein. Each pH was studied in triplicate.

The samples at pH 7.0, 8.0 and 9.0 containing lysozyme with CQA were also analysed by MALDI-TOF MS, as described previously, to detect possible covalent interactions. Blanks consisted of lysozyme at pH 7.0 and 9.0 without CQA.

3. RESULTS

3.1 Binding affinity of CQA for proteins at pH 7.0

The binding of CQA to BSA was investigated by three methods (equilibrium dialysis, the Hummel and Dreyer technique and ultrafiltration). These methods yielded quite identical results. The number of molecules of CQA bound per molecule of BSA (ν) at pH 7.0 (25^{o}C) was measured by the Hummel-Dreyer technique and by equilibrium dialysis at two different ionic strengths (**Figure 1**). The free CQA constituted more than 30% of the total CQA. This excess in free CQA was necessary to accurately determine the free ligand concentration, thereby avoiding a systematic error in the Scatchard plots (Nekhai et al., 1997). The concave shape of the Scatchard plots, representing the mechanism of CQA/BSA binding, seems to show a pattern of negative cooperativity; after the binding of one CQA molecule, the binding of another CQA molecule became more difficult. The increasing difficulty to bind more and

more CQA molecules may be due either to a change in protein tertiary structure by ligand binding, which reveals a negative cooperative mechanism (Koshland-Némethy-Filmer sequential model), or to the initial presence of classes of sites of different affinity (Wang and Pan, 1996). Circular dichroism (CD) experiments in the 270-310 nm region (no further data given) did not show any clear change in the tertiary structure of BSA in presence of CQA. Thus, the increasing difficulty to bind additional CQA molecules is likely due to the initial presence of different affinity classes of non-interacting sites on BSA, rather than to a structural change of BSA. However, the absorbance of CQA led to a high background noise in the CD spectra. Thus a cooperative mechanism (resulting from a structural change) cannot be totally excluded, especially for the lower affinity binding sites.

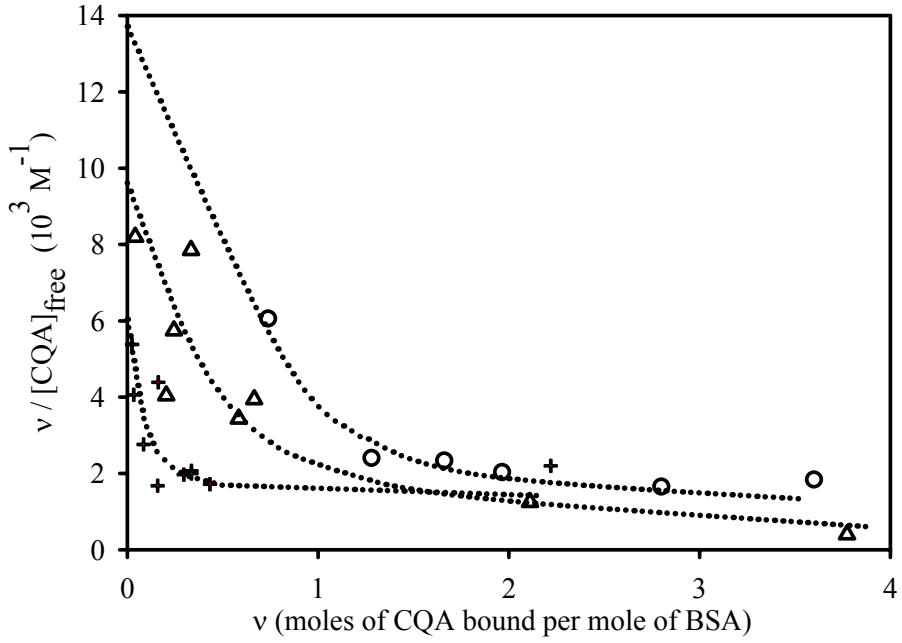


Figure 1. Scatchard plot for the binding of CQA to BSA (pH 7.0, 25°C), using different techniques: (△) Hummel-Dreyer (I = 0.062 M); (+) Hummel-Dreyer (I = 0.184 M); (○) equilibrium dialysis (I = 0.062 M). The dotted lines were obtained from the fitting models for two sets of binding sites.

The Scatchard plots were analysed with a graphic parameter fitting for the simplest model of several affinity classes, i.e. two classes of binding sites. At an ionic strength of 0.062 M, BSA contained one site of medium affinity with binding constants of $13x10^3$ +/- $2x10^3$ M^{-1} and $16x10^3$ +/- $5x10^3$ M^{-1}, according to the results of the Hummel-Dreyer technique and equilibrium dialysis experiments, respectively. The other BSA binding sites had a very low affinity for CQA. Because of this low affinity, the exact number of binding sites for the

second set of sites was difficult to determine. At 0.184 M ionic strength, the scattering of the points resulted in a non-precise estimation of the binding constant. One binding site with an affinity of $52x10^3$ +/- $25x10^3$ M^{-1} was present, whereas the other binding sites had a very low affinity for CQA (**Figure 1**).

The binding of CQA to BSA was also studied by isothermal titration calorimetry (ITC) (**Figure 2**). The slope of the curve was not steep, indicating that not all added ligand was bound, thus that the binding was moderately tight (Wiseman et al., 1989). The ITC data analysis software allowed the testing of three models of interactions: one set of sites, two sets of independent sites, or multiple interacting sites. The best model for fitting the curves (with randomised errors) was that of at least two sets of interacting binding sites (**Figure 2**). However, ITC alone cannot distinguish truly interacting sites from sites with differing binding constants (except if the sites of the protein are known to be identical). Thus, according to the ITC results, the affinity of BSA for CQA was the result of either at least two sets of interacting binding sites, or more than two sets of independent sites. No unambiguous value of the binding constant could be extracted from ITC data as the estimation of the binding constants led to several outcomes with similar error.

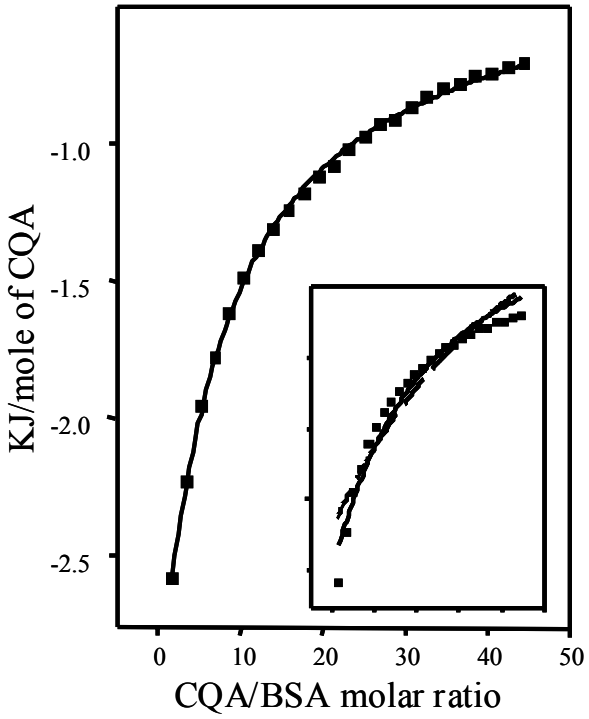


Figure 2. Isothermal titration calorimetry of CQA/BSA interactions (25°C): (■) data points; (—) fitting model for two sets of interacting sites. In the insert: (—) one set of sites; (- -) two sets of independent sites.

3.2 Reversibility of CQA-protein interactions

To determine how fast the interactions between CQA and protein were occurring, and to prove that covalent interactions were absent, gel filtration chromatography and MALDI-TOF MS experiments were performed. After incubation of 3-4 moles of CQA per mole of BSA for five minutes (pH 7.0, I = 0.062 M), a CQA/BSA complex could already be detected by gel filtration chromatography, and the amount of complex formed (as estimated from the absorbance of CQA at 324 nm) did not increase upon longer incubation. Thus, the interactions between CQA and BSA occurred within five minutes.

To verify that covalent protein-CQA complexes were absent, samples of lysozyme and α-lactalbumin incubated at a high CQA-protein ratio were analysed by MALDI-TOF MS (mass spectrometry). The analysis showed that lysozyme and α-lactalbumin did not form covalent complexes with CQA at pH 7.0 without incorporating air by stirring the solutions (data not shown). BSA was not analysed in this way since the lack of sensitivity of MALDI-TOF MS for high molecular weight proteins prevented the detection of covalent modifications of BSA. However, after extensive dialysis of BSA incubated with CQA, no CQA could be detected anymore in the sample according to absorbance measurements, indicating that the interactions were totally reversible.

3.3 Effect of low pH on CQA - protein interactions

The binding of CQA to BSA at low pH was investigated in order to determine which kind of non-covalent interactions play a role in the interactions between CQA and proteins. The number of CQA molecules bound per BSA molecule was slightly higher at pH 3.0 than at pH 7.0 at intermediate ratios (10-15 moles of CQA / mole of BSA (**Figure 3**)).

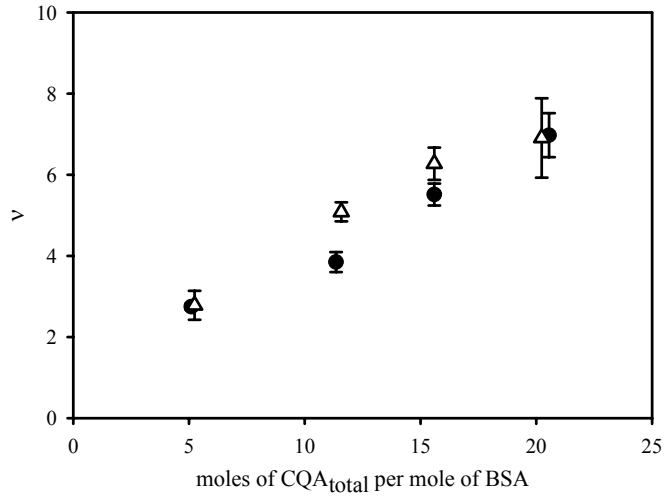


Figure 3. Number of molecules of CQA bound per molecule of BSA (ν) as a function of the number of moles of CQA added per mole of BSA at different pHs (I = 0.184 M, 25 °C; CQA, from 0.033% to 0.1 % (w/v)) as determined using ultrafiltration: (△) pH 3.0; (●) pH 7.0.

3.4 Effect of temperature and protein denaturation on CQA - protein interactions

The amounts of CQA bound to BSA at various temperatures (5, 25 and 60 °C) were measured by the Hummel-Dreyer technique (Table 1), concerning the higher affinity binding site. The number of molecules of CQA bound per molecule of BSA decreased when the temperature increased (Table 1).

The amount of CQA bound to heat denatured BSA and α-lactalbumin was studied using ultrafiltration. Lysozyme was not studied by ultrafiltration because of its low solubility after heat denaturation, whereas BSA and α-lactalbumin remained soluble after denaturation. The efficiency of the denaturation was monitored by DSC. BSA was entirely denatured. A peak of α-lactalbumin, which represented 5% of the enthalpy of the unheated protein, was still observed after heating, probably due to "renaturation" of α-lactalbumin (Fang and Dalgleish, 1998). Heated samples were applied on a gel filtration column. Nearly all BSA and α-lactalbumin molecules were aggregated. The aggregates were on average composed of about 14 molecules (900 kDa) and 21 molecules (300 kDa) of BSA and α-lactalbumin, respectively as estimated by their apparent molecular weight. The number of molecules of CQA bound to BSA decreased by 50 % when BSA was denatured (Table 1). The number of molecules of CQA bound to α-lactalbumin did not significantly change when α-lactalbumin was denatured (Table 1).

Table 1. Number of molecules of CQA bound per molecule of protein (ν) at pH 7.0 for BSA, α-lactalbumin and lysozyme, at various conditions and at different CQA-protein molar ratios using the Hummel-Dreyer technique (HD) or the ultrafiltration technique (UF).

Technique	Protein	Temperature	Concentration (mM CQA)	Molar ratio	ν
HD	BSA	25 °C	0.02[a]	0.12	0.04
HD	BSA	60 °C	0.02[a]	0.01	0.01
HD	BSA	25 °C	0.06[a]	0.52	0.18
HD	BSA	60 °C	0.06[a]	0.37	0.03
HD	BSA	5 °C	0.12[a]	1.00	0.34
HD	BSA	25 °C	0.12[a]	0.87	0.22
HD	BSA	60 °C	0.12[a]	0.74	0.08
HD	BSA	20 °C	0.17[a]	1.65	0.66
HD	Lysozyme	20 °C	1.69[a]	1.65	0.10
UF	BSA native	25 °C	0.20[a]	10	0.8 ± 0.1
UF	BSA heat denatured	25 °C	0.20[a]	10	0.4 ± 0.2
UF	α-lactalbumin native	25 °C	0.93[a]	10	0.4 ± 0.3
UF	α-lactalbumin heat denatured	25 °C	0.93[a]	10	0.45 ± 0.08

[a] For HD, the parameter which can be controlled (and indicated in the table) is the concentration of CQA in the elution buffer (CQA_{free}), whereas the indicated concentration for UF is the total concentration of CQA.

3.5 Effect of CQA on protein heat denaturation

The denaturation temperature of BSA, lysozyme and α-lactalbumin was measured as a function of CQA concentration using DSC (**Figure 4**).

In the presence of $3.7x10^3$ moles of CQA per mole of BSA, the denaturation temperature increased by 6 °C, while the denaturation enthalpy of BSA increased by about 700 kJ/mole (**Figure 4A**). The change in denaturation temperature for lysozyme and α-lactalbumin due to the presence of CQA was not as pronounced as for BSA (**Figure 4B**). A decrease of 1 °C in the denaturation temperature of lysozyme required the presence of 200 moles of CQA per mole of lysozyme (**Figure 4B**) while the denaturation enthalpy (330 kJ / mole of lysozyme) was not significantly changed at this molar ratio. At the same ratio, the denaturation temperature of α-lactalbumin was not significantly different from that without CQA (**Figure 4B**). The denaturation enthalpy of α-lactalbumin (270 kJ / mole) was also not significantly modified with increasing CQA concentration

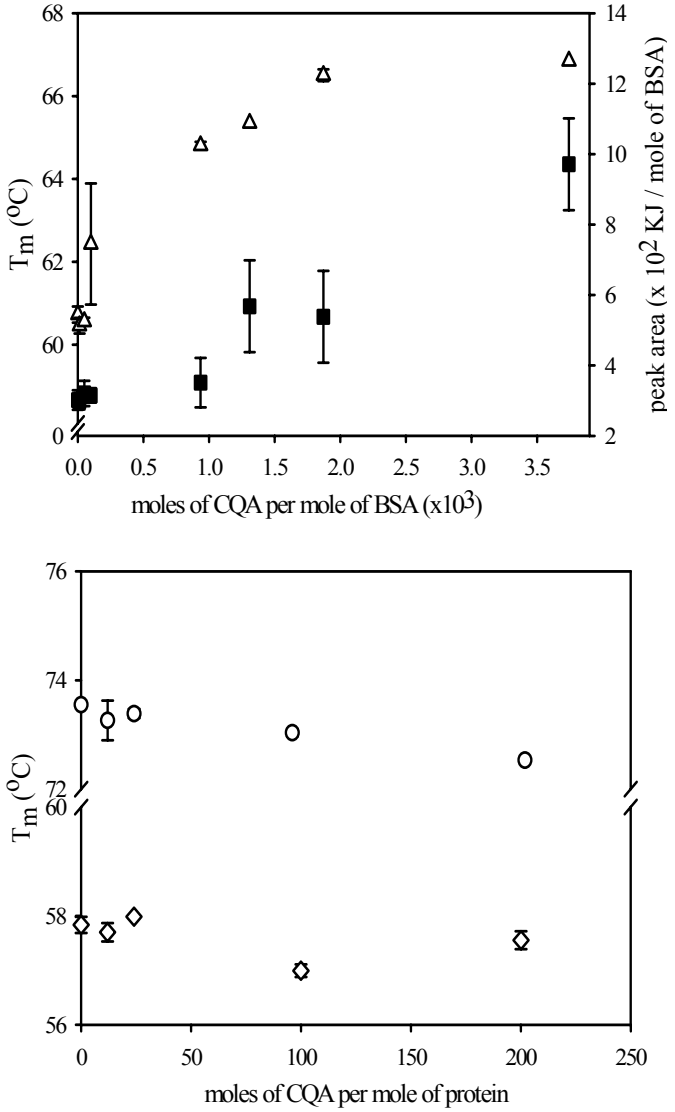


Figure 4. Denaturation temperature and enthalpy as a function of the number of moles of CQA present in solution per mole of protein (pH 7.0), as determined by DSC: (A) (△) denaturation temperature of BSA; (■) heat flow of BSA; (B) (○) denaturation temperature of lysozyme; (◇) denaturation temperature of α-lactalbumin.

3.6 Effect of CQA on protein solubility

The solubility, as a function of pH, of BSA, α-lactalbumin and lysozyme was investigated in the presence of 100 moles of CQA per mole of protein. The solubility of BSA (pI 4.7-4.9) and α-lactalbumin (pI 4.2-4.5) was not significantly affected by the presence of CQA (**Figure 5**). However, CQA significantly decreased the solubility of lysozyme (pI 10.5-11.3) at pH ≥ 8.0. Since at elevated pHs CQA may be oxidised by auto-oxidation leading to formation of reactive quinones, which may interact covalently with proteins, the presence of covalent interactions was investigated at pH ≥ 7.0. According to MALDI-TOF MS (**Figure 6**), the

covalent addition corresponded to two molecules of CQA (680-690 Daltons) per molecule of lysozyme at pH 8.0 and more than two at pH 9.0, whereas no such event occurred at pH 7.0.

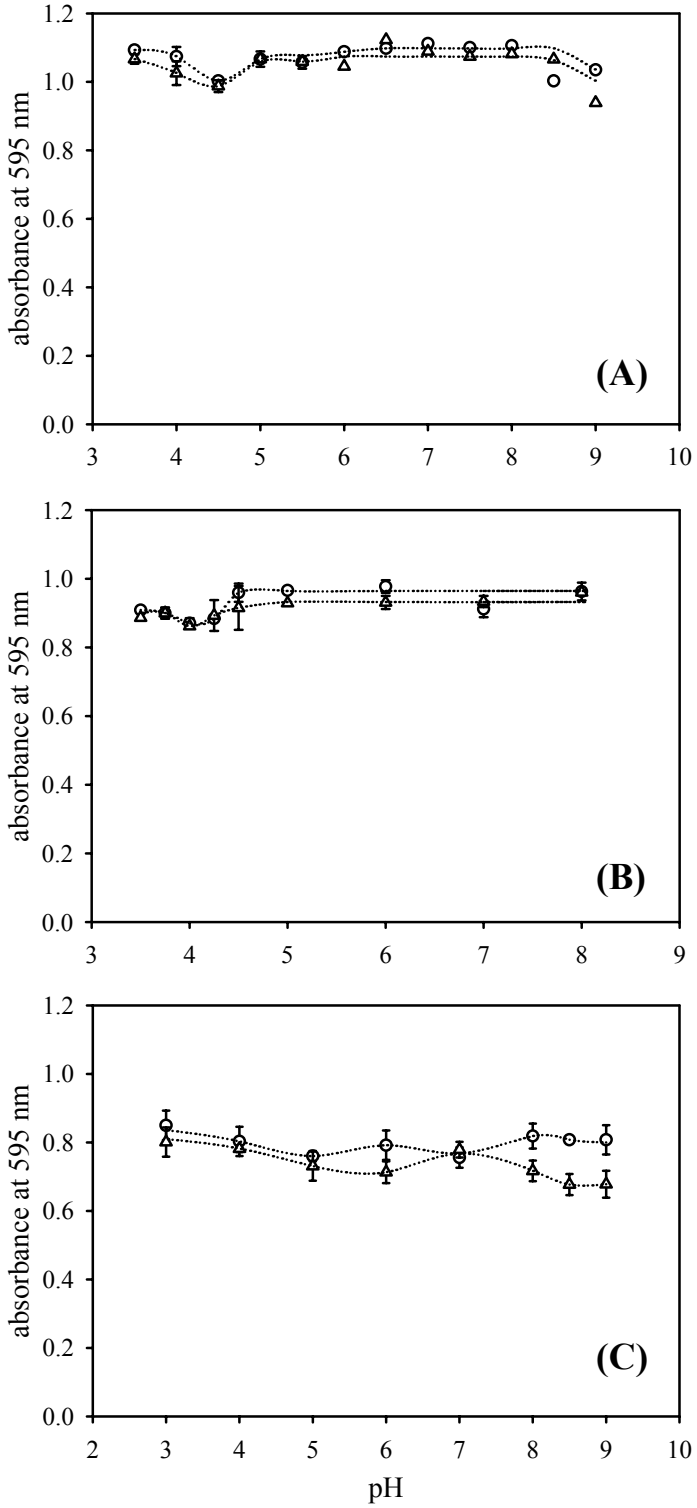


Figure 5. Protein solubility as a function of pH in the absence (○), and in the presence of 100 moles of CQA per mole of protein (△): (A) BSA (1 mg/ml); (B) α-lactalbumin (1 mg/ml); (C) lysozyme (2 mg/ml).

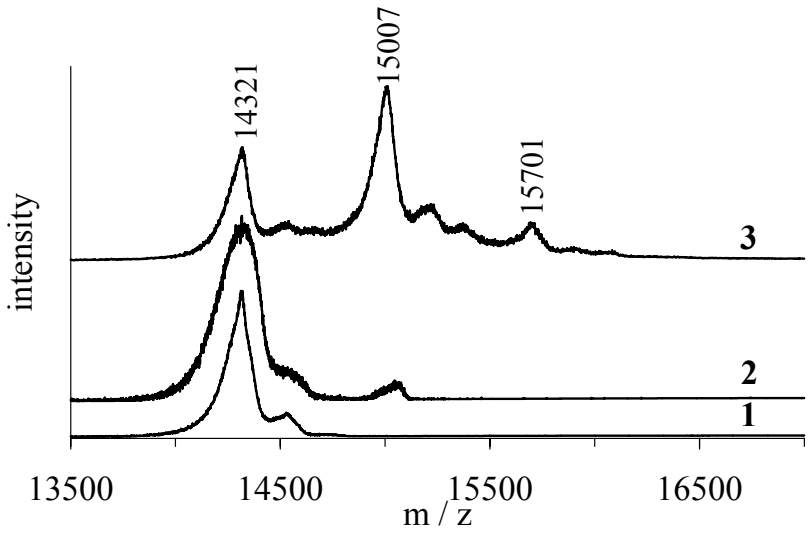


Figure 6. Mass spectra of lysozyme in the presence of 100 moles of CQA per mole of protein, at pH 7.0 (1), 8.0 (2) and 9.0 (3).

4. DISCUSSION

4.1 Characterisation of non-covalent interactions between CQA and proteins

Using equilibrium dialysis, the Hummel-Dreyer technique and ITC, it was shown that at pH 7.0 the affinity of BSA for CQA was the result of one site of medium affinity (13-16 x 10^3 M^{-1} at I= 0.062 M) and various sites of low affinity. These interactions resulted in an increasing difficulty to bind additional CQA, which was likely due to the initial presence of sites of different affinity, rather than to a negative cooperativity. In contrast to this, the interactions observed between CQA and 11S sunflower protein were the result of four binding sites with a similar binding constant of 4.5 x 10^3 M^{-1}, and showed a positive cooperativity (Shamanthaka Sastry and Narasinga Rao, 1990).

In Table 1, the amount of CQA bound to BSA and lysozyme at pH 7.0 were compared as a function of the free CQA concentration by the technique of Hummel and Dreyer. The lysozyme concentration used was higher than the BSA concentration used, because of the lower affinity of lysozyme for CQA compared to BSA. The number of molecules of CQA bound per molecule of protein at pH 7.0 was clearly smaller for lysozyme than for BSA, and seemed slightly lower for α-lactalbumin than for BSA (Table 1). Differences among proteins in the amount of binding could be explained by different facts, such as the amino acid

composition, e.g. the number of proline residues (Baxter et al., 1997), the value of the isoelectric point (Naczk et al., 1996) and the difference in average hydrophobicity (BSA > α-lactalbumin > lysozyme) according to several spectroscopic methods (Li-Chan, 1990).

Increasing the ionic strength increased the binding strength between CQA and BSA. Lowering the pH from 7.0 to 3.0 slightly increased the binding at certain CQA/protein ratios, without pronounced effects on the binding constants. As more than one CQA was bound per molecule of protein, this modification seemed to concern the low affinity binding sites. Furthermore, as lowering the pH from 7.0 to 3.0 had no effect on the binding constants, electrostatic interactions with amino acids having a sidegroup with a pKa between 7.0 and 3.0 do not seem to play a role in the interactions between CQA and BSA. Shamanthaka Sastry and Narasinga Rao (Shamanthaka Sastry and Narasinga Rao, 1990) observed that increasing the pH from pH 4.0 to pH 7.0 reduced the total number of CQA binding sites on the sunflower 11S protein, without affecting the binding constants.

Increasing the temperature strongly decreased the amount of CQA bound to BSA, even at temperatures where BSA was not heat denatured. This could indicate that CQA interactions are not dominated by hydrophobic interactions, as these increase upon heating. Hydrophilic interactions (hydrogen bonds, Van der Waals interactions, electrostatic interactions) decrease with increasing temperature (Relkin, 1996). Shamanthaka Sastry and Narasinga Rao (Shamanthaka Sastry and Narasinga Rao, 1990) concluded from the effect of temperature on the binding affinity and the number of binding sites that CQA seemed to interact with 11S sunflower protein by hydrogen bonding rather than by hydrophobic interactions. We can, however, not conclude from our results that hydrophobic interactions do not play a role in the interactions between CQA and BSA.

The denaturation of BSA decreased the number of moles of CQA bound to BSA by 50 %, whereas the denaturation of α-lactalbumin did not significantly modify the number of CQA molecules bound. However, denaturation produced aggregates of proteins, composed on average of about 14 molecules of BSA, and 21 molecules of α-lactalbumin. When the proteins are considered as spheres with surface = $4\ \pi$ x radius^2, and volume =$4\ \pi$ x radius^3 / 3, and according to the formula (volume^3) = 1.27 x protein molecular weight (Creighton, 1993), the surface of an aggregate of 14 molecules of BSA, and 21 molecules of α-lactalbumin, would be 60 %, and 65 %, smaller than the surface of separate 14 monomers of BSA, and 21 separate monomers of α-lactalbumin, respectively. The decrease of the binding of CQA to the

denatured BSA compared to the native BSA can thus be explained by the decrease of the total protein surface induced by the aggregation of BSA. In contrast to this, denatured α-lactalbumin seems to bind more CQA than the native α-lactalbumin, which could be explained by a difference in amino acid exposure at the surface of the protein.

4.2 Effect of non-covalent interactions with CQA on protein functional properties

At high CQA/protein ratios, CQA increased the denaturation temperature of BSA and the denaturation enthalpy of BSA. This may indicate a stabilisation of BSA against heat denaturation due to a stronger binding of CQA to the native protein than to the denatured protein. The denaturation temperature, however, continued to increase even when BSA should be considered fully occupied with ligand. Then, according to Cooper (Cooper et al., 2001), the stabilisation does not result from extra ligand binding, but from the additional free energy necessary to remove the ligand before the unfolding of the protein, as well as from the entropy of mixing of CQA (released into the bulk solution) that decreases with increasing concentrations of ligand in the bulk solution.

The presence of CQA slightly decreased the denaturation temperature of lysozyme. If a ligand binds preferentially to the unfolded form of a protein rather than to the native form, then the protein will be destabilised and unfolding will be encouraged (Cooper et al., 2001). This may indicate that unfolded lysozyme binds to CQA with a slightly higher affinity than native lysozyme. CQA did not significantly modify the denaturation temperature of α-lactalbumin.

The presence of CQA with α-lactalbumin and lysozyme during heat denaturation at pH 7.0 induced a covalent addition of 680-690 Daltons, according to MALDI-TOF MS experiments (data not shown). This modification is probably due to the thermal oxidation of phenols into quinones, which then react covalently with proteins. Such a reaction was shown to occur between quinones and amino, sulfhydryl, thioether, phenolic, indole and imidazole groups of proteins (Matheis and Whitaker, 1984). An addition of 680-690 Daltons corresponds to the addition of two molecules of CQA modified into a quinone (Rawel et al., 2000). Since only additions of two molecules of CQA, but no addition of monomeric CQA, were detected, it can be expected that two molecules of CQA react by condensation, prior to the interaction with proteins. Such dimers can be formed by coupled oxidation between a quinone and another phenolic molecule (Robards et al., 1999). If the mechanism proposed by Namiki and colleagues (Namiki et al., 2001) is followed, the binding of one dimer of CQA will lead to an

addition of 683 Daltons. The observed addition of 680-690 Daltons during heating and at alkaline pH seems thus to indicate a dimerisation of CQA prior to the interaction with proteins.

Also, the slight decrease in solubility of lysozyme at high CQA/protein ratios and basic pH's can be attributed to the oxidation, in alkaline solution, of CQA into quinones. In contrast to the solubility of lysozyme, the solubility of BSA and α-lactalbumin was not decreased by the presence of CQA at alkaline pHs. Covalent interactions created at alkaline pH with CQA have been observed not to modify the solubility of BSA (Rawel et al., 2002) and α-lactalbumin at these pHs (data not shown). The differences between lysozyme and BSA and α-lactalbumin may be caused by the large differences in isoelectric point between the proteins. The non-covalent interactions with phenolic compounds may precipitate proteins by a multidentate mechanism (by interacting simultaneously with several proteins), or by covering the protein with a less hydrophilic monolayer (Haslam, 1989). However, CQA is only monodentate, and BSA, α-lactalbumin and lysozyme seem to have a too low affinity for CQA to induce precipitation via a non-covalent way.

This study shows that only at very high molar CQA/protein ratios (≥100) large effects on protein properties can be observed like increasing the unfolding temperature by several degrees. In foods these ratios are generally much lower. For defatted sunflower meal e.g., which can be considered one of the richest sources of phenolic compounds, the molar ratio of molecules of CQA per mole of protein (hexamer of helianthinin) is only 1.3 (calculated from Gonzalez-Perez et al., 2002). In conclusion, when it interacts non-covalently, CQA does not have pronounced effects on the functional properties of globular proteins in food systems. However, the quinones from CQA, via their covalent interactions with proteins, are expected to have more pronounced effects on the functional properties of globular proteins.

LITERATURE CITED

Artz, W. E.; Bishop, P. D.; Dunker, A. K.; Schanus, E. G.; Swanson, B. G. Interaction of synthetic proanthocyanidin dimer and trimer with bovine serum albumin and purified bean globulin fraction G-1. *J. Agric. Food Chem.* **1987**, *35*, 417-421.

Bartolomé, B.; Estrella, I.; Hernández, M. T. Interaction of low molecular weight phenolics with proteins (BSA). *J. Food Sci.* **2000**, *65*, 617-621.

Bate-Smith, E. C.; Swain, T. *Comparative Biochemistry*; Academic Press: New York, United States, 1962; Vol. 3, p. 764.

Baxter, N. J.; Lilley, T. H.; Haslam, E.; Williamson, M. P. Multiple interactions between polyphenols and a salivary proline-rich protein repeat result in complexation and precipitation. *Biochemistry* **1997**, *36*, 5566-5577.

Boyes, S.; Strubi, P.; Dawes, H. Measurement of protein content in fruit juices, wine and plant extracts in the presence of endogenous organic compounds. *Lebensm. -Wiss.u.-Technol.* **1997**, *30*, 778-785.

Busch, M. H. A.; Kraak, J. C.; Poppe, H. Principles and limitations of methods available for the determination of binding constants with affinity capillary electrophoresis. *J. Chromatogr. A* **1997**, *777*, 329-353.

Clifford, M. N. Review. Chlorogenic acids and other cinnamates - nature, occurrence, dietary burden, absorption and metabolism. *J. Sci. Food Agric.* **2000**, *80*, 1033-1043.

Cooper, A.; Nutley, M. A.; Wadood, A. *Protein-Ligand Interactions: hydrodynamics and calorimetry. A practical approach*; Harding, S.E.; Chowdhry, B.Z., Eds.; Oxford University Press: Oxford, England, 2001, 287-318.

Creighton, T. E. *Proteins: structures and molecular properties. Second edition*; Freeman, W.H. and Company; Library of Congress Cataloging-in-Publication Data: New York, United States, 1993, p. 507.

Fang, Y.; Dalgleish, D. G. The conformation of α-lactalbumin as a function of pH, heat treatment and adsorption at hydrophobic surfaces studied by FTIR. *Food Hydrocolloids* **1998**, *12*, 121-126.

Gonzalez-Perez, S.; Merck, K. B.; Vereijken, J. M.; Van Koningsveld, G. A.; Gruppen, H.; Voragen, A. G. J. Isolation and characterization of undenatured chlorogenic acid free sunflower (*Helianthus annuus*) proteins. *J. Agric. Food Chem.* **2002**, *50*, 1713-1719.

Haslam, E. *Plant polyphenols: vegetable tannins revisited*; Phillipson, J.D.; Ayres, D.C.; Baxter, H., Eds.; Cambridge University Press: Cambridge, 1989, 167-192.

Hummel, J. P.; Dreyer, W. J. Measurement of protein-binding phenomena by gel filtration. *Biochim. Biophys. Acta* **1962**, *63*, 530-532.

Li-Chan, E. Hydrophobicity in food protein systems. In *Encyclopedia of food science and technology*; Y. H. Hui, Ed.; Wiley-Interscience: New York, 1990, 1429-1439.

Matheis, G.; Whitaker, J. R. Modification of proteins by polyphenol oxidase and peroxidase and their products. *J. Food Biochem.* **1984**, *8*, 137-162.

Murray, N. J.; Williamson, M. P.; Lilley, T. H.; Haslam, E. Study of the interaction between salivary proline-rich proteins and a polyphenol by ^{1}H-NMR spectroscopy. *Eur. J. Biochem.* **1994**, *219*, 923-935.

Naczk, M.; Oickle, D.; Pink, D.; Shahidi, F. Protein precipitating capacity of crude canola tannins: effect of pH, tannin, and protein concentrations. *J. Agric. Food Chem.* **1996**, *44*, 2144-2148.

Namiki, M.; Yabuta, G.; Koizumi, Y.; Yano, M. Development of free radical products during the greening reaction of caffeic acid esters (or chlorogenic acid) and a primary amino compound. *Biosci. Biotechnol. Biochem.* **2001**, *65*, 2131-2136.

Nekhai, S. A.; Beletzkij, V. E.; Graifer, D. M. Influence of systematic error on the shape of the Scatchard plot of $tRNA^{Phe}$ binding to eukaryotic ribosomes. *Biochem. J.* **1997**, *325*, 401-404.

O'Connell, J. E.; Fox, P. F. Effect of phenolic compounds on the heat stability of milk and concentrated milk. *J. Dairy Res.* **1999**, *66*, 399-407.

Privalov, P. L. Stability of proteins. Small globular proteins. *Adv. Protein Chem.* **1979**, *33*, 167-241.

Rawel, H. M.; Kroll, J.; Riese, B. Reactions of chlorogenic acid with lysozyme: physicochemical characterization and proteolytic digestion of the derivatives. *J. Food Sci.* **2000**, *65*, 1091-1098.

Rawel, H. M.; Rohn, S.; Kruse, H.-P.; Kroll, J. Structural changes induced in bovine serum albumin by covalent attachment of chlorogenic acid. *Food Chem.* **2002**, *78*, 443-455.

Relkin, P. Thermal unfolding of β-lactoglobulin, α-lactalbumin, and bovine serum albumin. A thermodynamic approach. *Crit. Rev. Food Sci. Nutr.* **1996**, *36*, 565-601.

Robards, K.; Prenzler, P. D.; Tucker, G.; Swatsitang, P.; Glover, W. Phenolics compounds and their role in oxidative processes in fruits. *Food Chem.* **1999**, *66*, 401-436.

Sarker, D. K.; Wilde, P. J.; Clark, D. C. Control of surfactant-induced destabilization of foams through polyphenol-mediated protein-protein interactions. *J. Agric. Food Chem.* **1995**, *43*, 295-300.

Scatchard, G. The attraction of proteins for small molecules and ions. *Ann. N. Y. Acad. Sci.* **1949**, *51*, 660-672.

Shamanthaka Sastry, M. C.; Narasinga Rao, M. S. Binding of chlorogenic acid by the isolated polyphenol-free 11S protein of sunflower (*Helianthus annuus*) seed. *J. Agric. Food Chem.* **1990**, *38*, 2103-2110.

Siebert, K. J. Effect of protein-polyphenol interactions on beverage haze, stabilization, and analysis. *J. Agric. Food Chem.* **1999**, *47*, 353-362.

Van Koningsveld, G.; Gruppen, H.; de Jongh, H. J.; Wijngaards, G.; van Boekel, M. A. J. S.; Walstra, P.; Voragen, A. G. J. The solubility of potato proteins from industrial potato fruit juice as influenced by pH and various additives. *J. Sci. Food Agric.* **2002**, *82*, 134-142.

Wang, Z.-H.; Pan, X.-M. Kinetic differenciation between ligand-induced and pre-existent asymmetric models. *FEBS Lett.* **1996**, *388*, 73-75.

Weder, H. G.; Schildknecht, J.; Lutz, R. A.; Kesselring, P. Determination of binding parameters from Scatchard plots. Theoretical and practical considerations. *Eur. J. Biochem.* **1974**, *42*, 475-481.

Wiseman, T.; Williston, S.; Brandts, J. F.; Lin, L.-N. Rapid measurement of binding constants and heats of binding using a new titration calorimeter. *Anal. Biochem.* **1989**, *179*, 131-137.

Chapter 3

Covalent interactions between proteins and oxidation products of caffeoylquinic acid

Chapter 3

Covalent interactions between proteins and oxidation products of caffeoylquinic acid*

The covalent interactions between caffeoylquinic acid (chlorogenic acid, CQA) oxidised by polyphenol oxidase (PPO) at pH 6.0, and α-lactalbumin, lysozyme and bovine serum albumin (BSA) were characterized and compared to non-enzymatically induced modifications at alkaline pH (pH 9.0). The effects of these modifications on protein properties were examined. Both ways of modification seemed to result in protein modification mainly via dimeric rather than via monomeric CQA quinones. These modifications lead to a decrease in both the number of free primary amino groups and the isoelectric pH (pI) of the proteins. Modification with CQA only induced a low degree of protein dimerisation, which also occurred through the action of PPO alone. Modification drastically reduced the solubility of lysozyme over a broad pH range, whereas that of α-lactalbumin was strongly reduced only at pH-values close to its pI. The solubility of BSA was much less affected than that of the other proteins, and only at acidic pH. These results indicate that the same kind of modifications occurs at pH 6.0 as at pH 9.0 and that these modifications clearly change the functional properties of globular proteins.

KEYWORDS: phenolic compounds; quinone; polyphenol oxidase; lysozyme; α-lactalbumin; BSA; solubility; chlorogenic acid

*Submitted for publication

1. INTRODUCTION

Phenolic compounds may interact with proteins, not only in plants, e.g. as a defence mechanism (Felton et al., 1989), but also in plant based foods, e.g. creating a haze in beer (Siebert, 1999), and in the human body, e.g. in the perception of astringency (Charlton et al., 2002). In addition, depending on the nature of the phenolic compound and the protein, phenolic compounds may either impair or enhance enzymatic digestion of proteins (Kroll et al., 2003). The interactions between phenolic compounds and proteins may also have positive effects on protein functional properties in food products, by e.g. improving foam formation and stability in specific cases (Sarker et al., 1995), and the formation of cross-links in gelatin gels (Strauss and Gibson, 2004). Controlling the interactions between proteins and phenolics may, therefore, help to control the functionality of plant foods.

Phenolic compounds can covalently react with proteins in the form of a phenolic radical or in the form of a quinone. The first mechanism involves the formation of a tyrosine radical that, due to its phenolic structure, may react with a phenoxyl radical, as e.g. occurs in the peroxidase catalysed reaction between tyrosine-containing peptides and ferulic acid (Oudgenoeg et al., 2001). The second mechanism involves the enzymatic or non-enzymatic oxidation of phenolic compounds into quinones, which can then react with proteins. The enzymatic oxidation to quinones can be catalysed by polyphenol oxidase (PPO). Polyphenol oxidases are divided into catechol oxidases and laccases. Catechol oxidases, also called tyrosinases, can catalyse the oxidation of *o*-diphenols to *o*-quinones, by their catecholase activity. Furthermore, when they also possess cresolase activity, they are able to hydroxylate monophenols to *o*-diphenols, which can subsequently be oxidised to quinones. Quinones can also be enzymatically formed via laccases, which can oxidise a wide range of substrates including *p*-diphenols. Although in the presence of hydrogen peroxide, peroxidases (POD) may also catalyse the formation of quinones, they play only a minor role in enzymatic browning of foods compared to catechol oxidases (Richard-Forget and Gauillard, 1997). In addition, the formation of quinones may also occur without the help of an enzyme, e.g. increasing the pH to alkaline conditions induces the deprotonation of the phenolic hydroxyl group, leading to quinone formation. This method is used in food industry e.g. to produce ripe olives by treating them with dilute NaOH in order to oxidise caffeic acid and hydroxytyrosol (García et al., 1996). Other ways to form quinones make use of chemical agents, e.g. periodate (Harrison and Hodge, 1982).

Once quinones are formed, they may react with other phenolic molecules, leading to the formation of dimeric and higher molecular weight condensation products (Cheynier et al., 1988). In addition, they may react with proteins e.g. via the lysine residues of the latter (Pierpoint, 1969). The covalent modification of proteins by phenolic oxidation products generated at alkaline pH has been studied extensively by Kroll, Rawel and co-workers (Kroll et al., 2003;Rawel et al., 2000;Rawel et al., 2001;Rawel et al., 2002). They showed that the reactions involve primary amino groups, thiol groups and tryptophan residues. They also demonstrated that covalent protein modification by phenols oxidised at alkaline pH induced protein cross-linking and a decrease of the isoelectric pH of the proteins (Kroll et al., 2003;Rawel et al., 2000;Rawel et al., 2001;Rawel et al., 2002).

In contrast to the effects of modification at alkaline pH, the effects of covalent protein modification with quinones at neutral or mildly acidic pH have not been investigated in detail. At these conditions, which are much more relevant to foods than alkaline pH, the oxidation of CQA usually occurs via catechol oxidases (PPO). In this paper, therefore, the modification of α-lactalbumin and lysozyme at moderate pHs is studied using a system in which quinones of caffeoylquinic acid (chlorogenic acid) are generated by the action of PPO. The results obtained with this system are compared with those obtained by modification at alkaline pH. α-Lactalbumin and lysozyme, and in some experiments bovine serum albumin (BSA), are used as representatives of globular food proteins. Using these proteins, the extent of PPO modification is characterised and the effects of these modifications on protein solubility and foam stability are studied.

2. MATERIAL AND METHODS

2.1 Materials

Bovine serum albumin (BSA, fractionated by cold alcohol precipitation and essentially fatty acid free), bovine α-lactalbumin (type I, holo-α-lactalbumin), lysozyme (from chicken egg white) and caffeoylquinic acid (CQA; 1',3',4',5'-tetrahydroxycyclohexanecarboxylic acid 3-(3,4)-dihydroxypropenyldihydroxyphenyl-1-propenoate) were purchased from Sigma Chemical Company (St Louis, MO, USA) and used without further purification. Tyrosinase from mushroom (EC 1.14.18.1, mPPO) was purchased from Fluka (Steinheim, Germany) and had an activity of 3216 U per mg (1 U oxidises 1 μmol of 4-methylcatechol per min at pH 6.5 and 25^{o}C).

Samples incubated with PPO (with or without CQA) are denoted PP samples, while the ones incubated at alkaline pH are denoted ALK samples.

2.2 Incubation of proteins with CQA and mPPO

Lysozyme [0.29% (w/v)] and α-lactalbumin [0.28% (w/v)] were incubated for 2.5 h with mPPO (10 U/ml) and 0.2% to 0.9% (w/v) of CQA in the case of α-lactalbumin and 0.1% to 0.4% (w/v) of CQA in the case of lysozyme in 0.1 M potassium phosphate buffer (pH 6.0; ionic strength (I) = 0.116 M) or 0.1 M potassium acetate buffer (pH 5.0; I = 0.068 M), by stirring in open beakers at 40^{o}C. The molar ratios of CQA to primary amino group (CQA/PAG) were 1, 2, 3 and 4 for α-lactalbumin (13 PAG per protein molecule), and 1, 2 and 3 for lysozyme (7 PAG per protein molecule). The reaction was stopped by addition of $NaHSO_3$ to reach a final concentration of 5 μM $NaHSO_3$. Next, samples were dialyzed in dialysis tubing with a molecular weight cut-off of 12-14 kDa against 5 μM $NaHSO_3$, and stored at –20^{o}C. The samples used for solubility experiments were dialyzed against 0.01 M potassium phosphate buffer (pH 7.0; I = 0.018 M) containing 5 μM $NaHSO_3$. Blanks consisted of protein, protein with CQA, protein with PPO, and CQA incubated with PPO.

2.3 Incubation of proteins with CQA at alkaline pH

BSA, α-lactalbumin and lysozyme were covalently modified at pH 9.0 by quinones of CQA, and subsequently dialysed and freeze-dried according to the protocol of Rawel and co-workers (Rawel et al., 2000). The final protein concentration was 1% (w/v). The CQA/PAG molar ratios were 0.3, 1, 2, 3 and 10 for lysozyme, 0.5, 1, 2, 3, 4 and 10 for α-lactalbumin, and 1 and 3 for BSA (59 PAG per molecule). Blanks consisted of proteins without CQA, and CQA without proteins.

2.4 Protein content

The nitrogen content of the samples was measured using the Dumas combustion method on a NA 2100 nitrogen and protein analyzer (ThermoQuest, Rodano, Italy) according to the instructions of the manufacturer. After calibration with urea or methionine, the protein concentration was deduced using the appropriate nitrogen-protein conversion factors (6.02 for BSA, 5.29 for lysozyme, and 6.25 for α-lactalbumin).

2.5 MALDI-TOF MS analysis

Matrix assisted laser desorption/ionization - time of flight mass spectrometry (MALDI-TOF MS) analysis was performed on samples solubilized in 0.2% (v/v) TFA following the drying droplet method with external calibration (**Chapter 2**). Samples were analysed in duplicate on an Ultraflex MALDI TOF MS (Bruker Daltonics, Bremen, Germany).

2.6 Free primary amino groups

The number of primary amino groups was estimated using the OPA (ortho-phtaldialdehyde) method of Church and co-workers (Church et al., 1983). Prior to the measurements, samples were incubated for 24h in 8 M urea. The absorbance of the sample in OPA reagent was corrected for the absorbance of the OPA reagent and for the absorbance of the sample in water (without OPA reagent). The protein concentration was measured by the Dumas method, as described above.

2.7 Gel filtration chromatography

Gel filtration chromatography was performed for α-lactalbumin [0.286% (w/v)] and lysozyme [0.284% (w/v)] modified at pH 6.0 using mPPO. Of the native and modified proteins, 20 µl were applied on a Shodex KW-803 column (8 x 300 mm; flow rate: 0.6 ml/min; Showa Denko Europe GmbH, Munich, Germany) and eluted using 40% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid (TFA) on a HPLC system (Thermo Separation Products Inc., Riviera Beach, FL). Protein elution was detected at 280 nm and CQA elution at 324 nm. A blank of CQA [0.18 % (w/v)] incubated with PPO at pH 6.0 was also analyzed. A calibration mixture (13.7 - 2,000 kDa, Amersham Pharmacia Biotech, Uppsala, Sweden) for molecular weight determination was applied in order to estimate the sizes of aggregated proteins.

2.8 Electrophoresis

SDS-PAGE was performed using gradient gels of 10-15% with a Pharmacia PhastSystem according to the instructions of the manufacturer (Amersham Pharmacia Biotech, Uppsala, Sweden). Depending on the intensity of the bands, the gels were colored using Coomassie Brilliant Blue and by Silver staining.

2.9 Capillary isoelectric focusing

Capillary isoelectric focusing (cIEF) was performed on a neutral capillary (21 cm, 50 μm I.D.) on a Beckman P/ACE^{TM} MDQ System (Beckman Coulter, Fullerton, USA). Freeze-dried proteins were incubated overnight in 8 M urea [1 % (w/v)]. The ampholyte gel and the anolyte and catholyte solutions were prepared using a Proteome Lab cIEF 3-10 kit (Beckman Coulter). The runs were performed according to the instructions of the manufacturer. The pI markers used were carbonic anhydrase II (pI 5.9) and β-lactoglobulin A (pI 5.1). Protein elution was detected at 280 nm.

2.10 Chromatofocusing

The iso-electric point of some modified proteins was also determined by chromatofocusing. α-Lactalbumin [0.67% (w/v)], which was covalently modified at alkaline pH at CQA/PAG molar ratios of 0, 1, 2 and 3, was dissolved in 0.025 M bis-Tris (pH 5.9) containing 1 M urea. After 24h, the samples were centrifuged (10 min, 11,000 x *g, 25*^{o}C). Aliquots (50 μL) were applied on to a mono P HR column (5 x 200 mm) that had been equilibrated with the above buffer. The elution was performed at a flow rate of 1 ml / min, on a ÄKTA Purifier system (Amersham Biosciences, Uppsala, Sweden). The unbound proteins were eluted with one column volume of the buffer used to dissolve the protein. To elute proteins possessing a pI between 5.9 and 3.0, nine column volumes of 5% (v/v) Polybuffer 74-HCl (Amersham Biosciences), pH 3.0, containing 1 M urea, were applied. The column was rinsed with 1 ml of 1 M NaCl after each run. Protein elution was detected at 280 nm, CQA elution at 324 nm.

2.11 Protein solubility

Modified and unmodified BSA, α-lactalbumin and lysozyme were dissolved (0.5% (w/v)) in 0.01 M potassium phosphate buffer (pH 7.0; I = 0.018 M). The protein solutions were subsequently adjusted to various pHs with NaOH or HCl (1 M or 6 M). After two hours, the samples were centrifuged (15 min, 12,700 x *g*, 25^{o}C). The protein concentration in the supernatant was measured using the Dumas combustion method, as described previously. Each pH was studied in triplicate.

2.12 Differential scanning calorimetry

Samples used for differential scanning calorimetry (DSC) experiments were BSA [0.2% (w/v)], α-lactalbumin [0.2% (w/v)] and lysozyme [0.3% (w/v)] that had been covalently

modified at alkaline pH at a CQA/PAG ratio of 1, and was subsequently dialysed and freeze-dried. Proteins, dissolved in 0.1 M potassium phosphate buffer (pH 7.0; I = 0.187 M), were injected in the sample cell of a VP-DSC calorimeter (MicroCal Inc., Northampton, MA) and analysed for their heat denaturation properties (**Chapter 2**). Each experiment was repeated three times. Blanks consisted of proteins incubated at pH 9.0 in the absence of CQA.

2.13 Air-water interfacial and foam properties

Samples (100 μl) of protein solutions [0.3% (w/v)], that had been modified at alkaline pH at CQA/PAG ratios of 0, 1 and 3, were applied on to a Superdex 75 PG gel filtration column (10 x 65 cm; flow = 35 ml/min; Amersham Biosciences, Uppsala, Sweden) and eluted with 17 mM potassium phosphate buffer (pH 7.0; I = 0.03 M) to remove protein aggregates and CQA-CQA aggregates. The fractions corresponding to protein monomers were collected. After estimation of the protein content, the surface tension and foam properties of these fractions were studied.

The surface tension of samples was determined using an automatic drop tensiometer (ADT-Tracker, I.T. Concept, Longessaigne, France). The shape of an air bubble in rising configuration in a protein solution [0.01% (w/v)] was digitally analysed. After 3600 sec, the elastic modulus was obtained during dynamic oscillation of the area of the bubble with an area deformation of 6.56%.

Foam was prepared according to the whipping method of Caessens and co-workers (Caessens et al., 1997). A protein solution [1% (w/v)] was whipped for 3 min at 3000 rpm and poured into a cuvette (45 x 57 x 134 mm). The mean bubble diameter (d_{21}) was determined by images made from the cuvette in reflection mode via a prism, every 60 seconds during 1h.

3. RESULTS

To characterise the effects of CQA modification at pH values relevant to food products, the proteins were covalently modified at mildly acidic pH (pH 5.0 and pH 6.0), using PPO. In order to be able to compare the results with published data (Rawel et al., 2000;Rawel et al., 2002), modification was also carried out at alkaline pH (pH 9.0). Subsequently, the modified proteins were studied with respect to the extent of modification, and the effects of modification on protein mass and on their functional properties. Upon modification, lysozyme, α-lactalbumin and BSA solutions, which were uncoloured in the unmodified state

(white when freeze-dried), became blue-green when modified at pH 9.0 and 6.0, or yellow when modified at pH 5.0. The modified proteins also showed absorbance at 320 nm, which was absent with the unmodified proteins. The blanks containing only oxidised CQA were brown, except when incubated with PPO at pH 5.0 (pale yellow).

3.1 Extent of protein modification

The mass of the proteins modified using PPO at various CQA/PAG ratios was measured by MALDI-TOF MS.

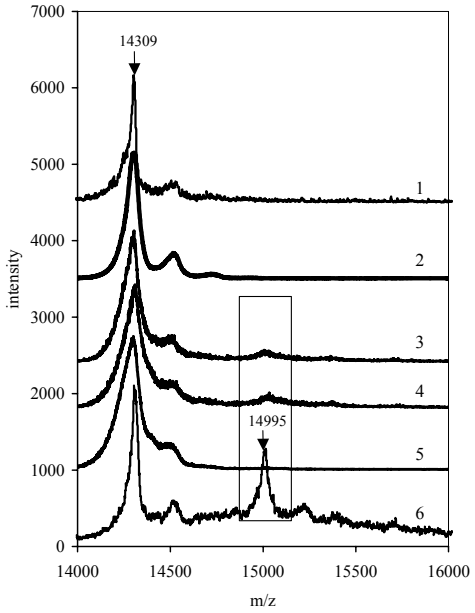


Figure 1. Mass spectra of lysozyme of unmodified lysozyme (1), lysozyme incubated with only PPO (2), lysozyme modified with PPO at pH 6.0 with CQA/PAG molar ratios of 2 (3) and 3 (4), at pH 5.0 with CQA/PAG molar ratio of 2 (5), and ALK modified lysozyme with CQA/PAG molar ratio of 2 (6).

The mass spectrum of unmodified lysozyme showed a peak at 14309 Da, which is in agreement with its sequence, and an additional peak at 14524 Da (**Figure 1**), which was also observed by Rawel and co-workers (Rawel et al., 2000). Modifications at CQA/PAG ratios of 2 and 3 in the presence of PPO at pH 6.0 show the presence of a peak around 14995 Da, corresponding to a covalent addition of 686 Da (**Figure 1**). This mass addition may correspond to the addition of a dimer of CQA (Namiki et al., 2001). No pronounced differences were found between the various CQA/PAG ratios (**Figure 1**). Similar to lysozyme, the mass of α-lactalbumin after incubation with CQA also showed modification via the addition of a compound with a mass similar to that of a CQA dimer (**Figure 2**, CQA/PAG = 1). For both proteins, no modification was observed after incubation with PPO at pH 5.0, as can be seen for lysozyme in **Figure 1**. Both methods of CQA oxidation were compared

concerning their effect on the molecular mass of α-lactalbumin and lysozyme. The mass difference due to ALK modification of lysozyme (**Figure 1**) and α-lactalbumin (data not shown) corresponded to a similar mass difference (686 Da) as that observed after PPO modification (pH 6.0).

To study the maximum attainable degree of modification, an incubation was carried out at alkaline pH with CQA/PAG = 10 (**Figure 2**). The covalent additions approximately corresponded to multiples of two molecules of CQA (686 Da) resulting in compounds with masses of 14995 Da and of 15209 Da. Additions of three and four molecules of CQA were also observed, giving compounds of 15345 and 15680 Da, respectively. These peaks, however, had a lower intensity than the peaks of the dimeric CQA modifications (**Figure 2**). While up to four molecules of CQA were detected per molecule of lysozyme, α-lactalbumin was mainly modified by only two molecules of CQA (**Figure 2**).

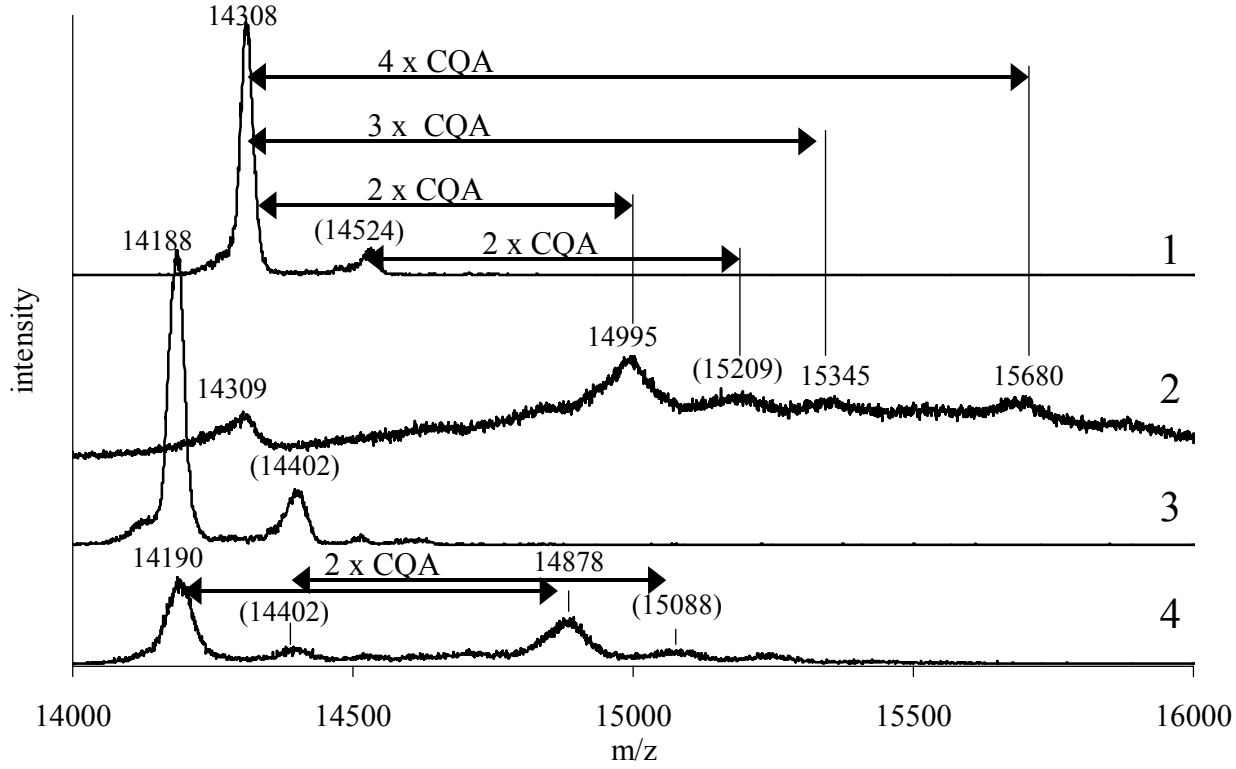


Figure 2. Mass spectra of ALK modified proteins: lysozyme unmodified (1) and modified with CQA/PAG =10 (2); α-lactalbumin unmodified (3) and modified with CQA/PAG =10 (4). Between brackets the masses of minor peaks and CQA modifications thereof are given.

In order to study the degree of protein modification, the number of modified primary amino groups per protein was estimated in proteins modified by CQA using PPO at pH 6.0 and at alkaline pH in the absence of PPO. The number of free PAG of α-lactalbumin (**Figure**

3) decreased linearly from 10 to 4 upon increasing the concentration of CQA. The number of free PAG measured after alkaline modification followed the same trend as that upon PPO modification (pH 6.0) (**Figure 3**). Up to three PAG of lysozyme were observed to be modified after PP and ALK modification (results not shown).

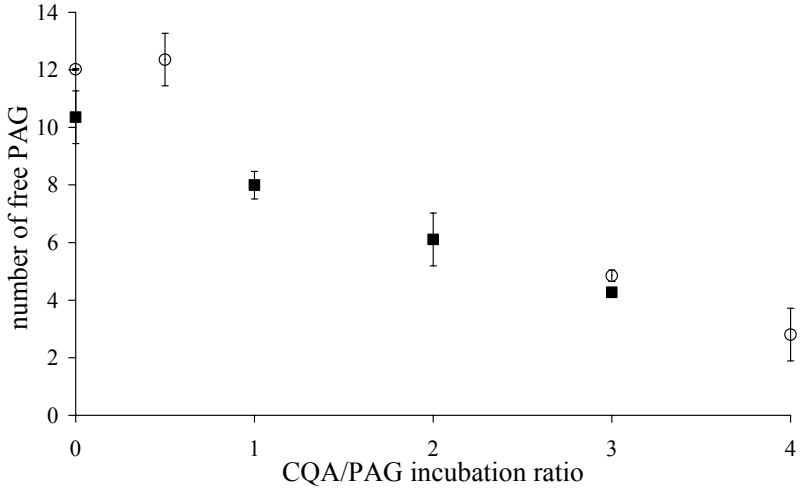


Figure 3. Number of free primary amino groups of α-lactalbumin as a function of the number of molecules of CQA per PAG, after modification via PPO (■) or at alkaline pH (○).

3.2 Effect of modification on protein cross-linking and isoelectric pH

Using gel filtration chromatography, dimers of lysozyme, which were absent after incubation with only PPO, were shown to be formed to a low extent (6% of the area of the protein monomer) in the presence of CQA and PPO, at pH 6.0 (**Figure 4**).

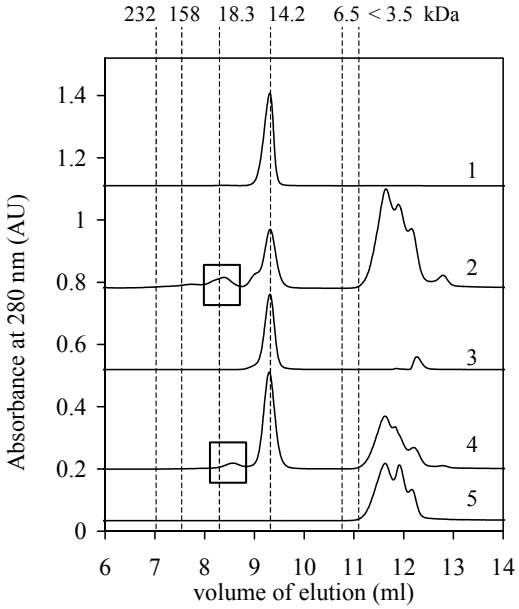


Figure 4. Gel filtration chromatograms of: α-lactalbumin unmodified (1), modified with PPO at CQA/PAG = 4 (2); lysozyme unmodified (3), modified with PPO at CQA/PAG =2 (4); CQA with PPO (5).

Using gel filtration chromatography, dimers of α-lactalbumin were also observed and represented 19% of the area of the protein monomer (Figure 4). Similar results were observed using SDS-PAGE, where smears of dimers were formed in addition to smears of monomers (results not shown). These dimers were also observed after incubation with only PPO, i.e. in the absence of CQA (data not shown).

A decrease of the pI is expected due to the loss of primary amino groups and the introduction of the carboxylic acid groups of CQA molecules. Capillary isoelectric focusing was used in order to be able to study a wide pH range for lysozyme. Whereas the main fraction of unmodified lysozyme was outside the pH range focused (pI > pH 9.5), incubation at a CQA/PAG molar ratio of 0.3 resulted in some fractions having a pI between 6.5 and 3.6 (**Figure 5**). When a CQA/PAG ratio of 1 was used, the proportion of protein with lower pIs ($4.6 \geq pI \geq 3.6$) increased. At a CQA/PAG ratio of 2, proteins with pIs lower than 3.0 were present (**Figure 5**). At the latter ratio, proteins with high pIs (pI >8.0) were still present in ALK samples, while they were absent from PP samples (**Figure 5**). This would indicate a higher degree of modification (or protein aggregation) in the presence of PPO compared to at alkaline conditions.

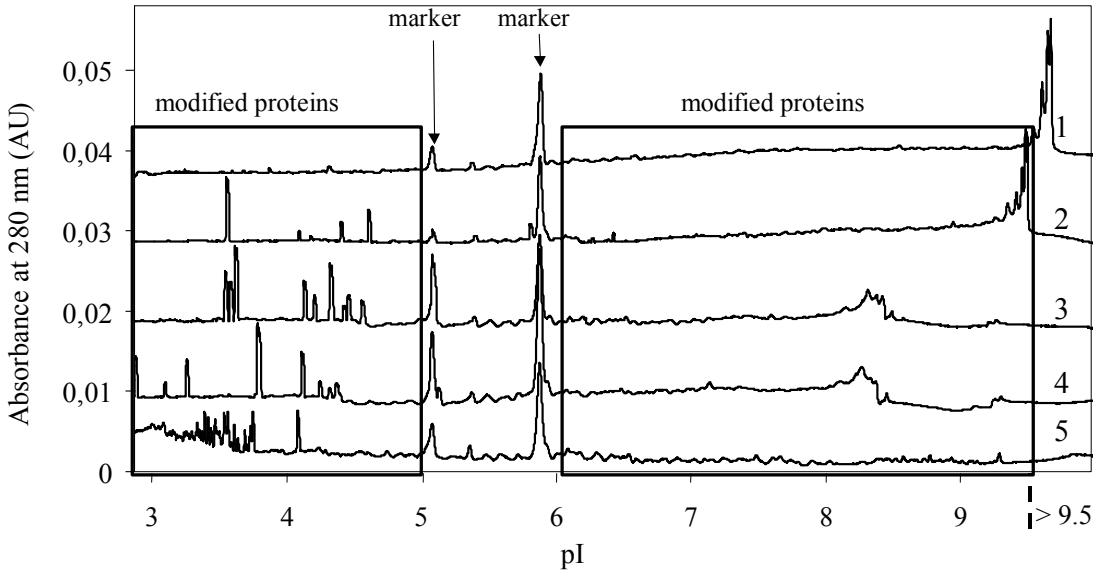


Figure 5. Capillary isoelectric focusing of lysozyme at various CQA/PAG ratios: (1) 0; (2) 0.3; (3) 1; (4) 2, all at ALK conditions. (5) CQA/PAG = 2, PPO modification.

Using chromatofocusing, unmodified α-lactalbumin was observed to possess a pI of 4.5, whereas ALK modified α-lactalbumin showed pI values of 3.9, 3.7 and 3.4 (data not shown). The pI of PP modified α-lactalbumin was not investigated.

3.3 Effect on protein functional properties

The effects of CQA modification on protein solubility, heat denaturation behaviour and foam properties were studied as well.

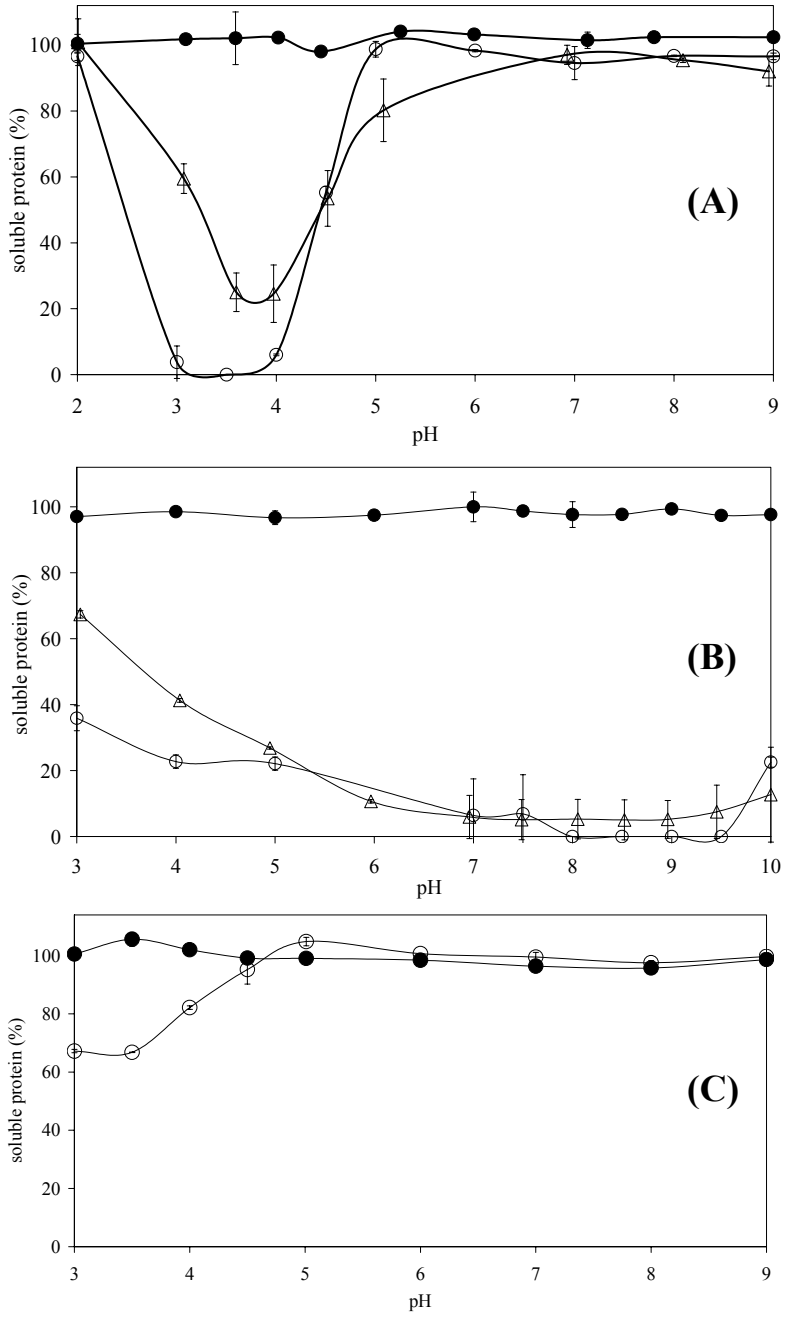


Figure 6. Protein (0.5% (w/v)) solubility as a function of pH: unmodified protein (●); PPO modification (△); ALK modification (○); CQA/PAG ratio of 2 for (A) α-lactalbumin and (B) lysozyme; and 1 for (C) BSA.

The effect of PPO modification on protein solubility as a function of pH was investigated after modification at a CQA/PAG ratio of 2 for α-lactalbumin and lysozyme. Modification with CQA drastically decreased the solubility of α-lactalbumin (**Figure 6A**) at pHs ≤ pI of the unmodified protein. In addition, modification with CQA induced a shift in the pH of minimum solubility of α-lactalbumin from 4-4.5 to 3.5-4.0 (**Figure 6A**). The decrease in the solubility of lysozyme (pI 10.5-11.3) took place over a much broader pH range (from pH 3.0 to pH 10.0, **Figure 6B**) than for α-lactalbumin. When the effects of PPO modification on solubility are compared with the effects of ALK modification, it can be concluded that both methods decreased the solubility of the proteins in a similar way and to a similar extent (**Figure 6 A and B)**. Because of the lack of solubility of modified lysozyme, it could not be used for foam studies. Therefore, BSA was introduced as an additional model protein. Modification at a CQA/PAG ratio of 1 for BSA (CQA/protein ratio = 60) significantly decreased the solubility of BSA at pH < 5, the pI of unmodified BSA (**Figure 6C**).

The denaturation temperatures of unmodified and ALK modified proteins after incubation at a CQA/PAG ratio of 1 were estimated using DSC. The denaturation temperature of BSA increased by 5^{o}C due to modification, whereas that of α-lactalbumin increased by 1^{o}C and that of lysozyme decreased by less than 1^{o}C (**Table 1**).

Table 1. Denaturation temperature of ALK modified proteins at CQA/PAG ratios of 0 and 1, as determined by DSC.

	average denaturation temperature (^{o}C)
α-lactalbumin	58.2 ± 0.3
α-lactalbumin, CQA / PAG = 1	60.6 ± 0.7
lysozyme	73.4 ± 0.2
lysozyme, CQA / PAG = 1	72.2 ± 0.4
BSA	60.4 ± 0.3
BSA, CQA / PAG = 1	65.8 ± 0.6

The CQA-CQA complexes and protein aggregates present were removed by gel filtration, in order to solely study the properties of CQA modified monomeric proteins. The effects of the ALK modification on the surface tension at the air-water interface and on the foam properties of BSA and α-lactalbumin were studied. Modification had neither an effect on the equilibrium surface tension, nor on the elastic modulus (**Table 2**). However, the initial mean bubble diameter of foam formed with α-lactalbumin seemed to decrease as a function of the degree of CQA modification, whereas that of BSA was not affected (**Table 2**). As monitored

by the mean bubble size with time (**Table 2**), no clear effect of the CQA/PAG ratio could be observed on the stability of foams made with BSA and with α-lactalbumin.

Table 2. Surface tension and elastic modulus at air-water interface and mean bubble diameter (d_{21}) of foam with unmodified or ALK modified protein [0.1% protein (w/v)] (pH 7.0; I=30 mM).

	CQA/PAG ratio	surface tension[a]	elastic modulus[a]	mean bubble diameter (μm)	
		(mN/m)	(mN/s)	0 s	1700 s
α-lactalbumin	0	53±1	52±4	275	161
	1	54±4	52±10	221	181
	3	54±2	45±13	206	150
BSA	0	55±1	53±3	188	249
	1	57±1	55±5	187	253
	3	57±1	58±1	192	220

[a] After 3600 s.

4. DISCUSSION

4.1 Modification at moderate pHs

From the MALDI-TOF MS results and from the smears observed in SDS PAGE (results not shown), it can be concluded that the modification of proteins by PPO oxidised CQA was effective and occurred already at low CQA/protein ratios when incubated at pH 6.0, but was limited at pH 5.0. When proteins were modified by CQA oxidised by periodate at pH 4.0 and pH 7.0, the same degree of protein modification was observed (results not shown). Therefore, the lower apparent reactivity of PPO oxidised CQA at pH 5.0 is most likely due to the lower activity of PPO at this pH.

Protein modification with quinones formed at pH 6.0 (PP) and pH 9.0 (ALK) resulted in a shift of the isoelectric pH to lower values causing the solubility of α-lactalbumin and lysozyme at pH ≤ pI to be strongly reduced, whereas the solubility of BSA was only slightly affected. These observations were also made by Rawel and colleagues after alkaline CQA modification of lysozyme and BSA (Rawel et al., 2000;Rawel et al., 2002).

MALDI-TOF MS results showed that, both upon PPO and ALK modification, lysozyme, in contrast to α-lactalbumin, was able to simultaneously react with CQA at more than one protein site. This higher degree of modification for lysozyme can explain the stronger decrease in pI and solubility observed with this protein. Although, α-lactalbumin and lysozyme possess very similar sequences, they differ in their isoelectric pH, as lysozyme has a

pI of 10.5-11.3 and α-lactalbumin a pI of 4.2-4.5. Because CQA possesses a pKa of 2.7, stronger electrostatic repulsion and lower electrostatic attraction between α-lactalbumin and CQA may be expected than with lysozyme, leading to more possibilities for interactions with lysozyme at all pHs.

4.2 Mechanism of covalent modifications

Using MALDI-TOF MS, it was shown that the reaction with PPO oxidised CQA induced modification of α-lactalbumin and lysozyme mainly by the addition of multiples of two molecules of CQA. This indicates a reaction primarily with dimers of CQA, whereas CQA monomers would be less reactive. To our knowledge, this is the first study that indicates that dimeric phenolic compounds are preferentially coupled to proteins. The addition of 686 Da, on α-lactalbumin and lysozyme, corresponds to the theoretical mass of 686 Da of a dimer formed by an oxidative coupling reaction between a quinone and a CQA molecule, via a mechanism proposed by Namiki and colleagues with another phenolic compound, ethyl caffeate (Namiki et al., 2001). When proteins were modified by CQA oxidised at alkaline pH, the main addition observed on the proteins also corresponded to the molecular mass of a dimer of CQA, indicating a similar mechanism at both conditions. The apparently higher reactivity of phenolic dimers compared to monomers may result from the fact that dimers generally have a lower redox potential than monomers (Singleton, 1987).

These interactions are likely to occur not only with primary amino groups, but also with the side-chains of cysteine (Felton et al., 1989), tryptophan (Rawel et al., 2002) and histidine (Hurrell et al., 1982) residues. Measuring the number of modified primary amino groups thus may gives only a limited indication of the extent of modification. On the 13 existing PAG, six PAG were modified in PP α-lactalbumin at pH 6.0, identical to the observation for ALK α-lactalbumin (**Figure 3**). These residues should be among lysine residues 13, 16, 58, 98, 108 and 114 and the N-terminal residue, which are all solvent exposed in α-lactalbumin (http://ebi.ac.uk/thornton-srv/databases). Up to three PAG of the seven existing PAG in lysozyme were modified after PP and ALK modification (results not shown), similar to the observations made by Rawel and co-workers (Rawel et al., 2000). Therefore, as observed for α-lactalbumin, PP and ALK modifications seem to result in the same degree of PAG modification. Of the reactive PAG of lysozyme, lysine 97 is likely to be modified, because of its presence at the surface (Fujita et al., 1995). Other PAG residues, which should

be able to react, are lysine residues 33, 96 and 116. In contrast to these residues, the N-terminal amino group is partially shielded, while lysine residues 1 and 13 seem also to be largely hidden (Fujita et al., 1995).

4.3 Effect on cross-linking

From the gel filtration chromatography results, it can be concluded that the reaction with PPO oxidised CQA induced a low degree of dimer formation in lysozyme. This dimerisation of lysozyme was also observed upon modification at alkaline pH (data not shown). Cross-linking induced by phenolic compounds has also been observed with CQA after PPO treatment of potato virus X (Pierpoint et al., 1977), and after alkaline treatment of proteins (Rawel et al., 2002). The occurrence of cross-linking can be explained by the reaction of one molecule of an oxidised phenolic compound with two amino acids (Kramer et al., 2001;Peñalver et al., 2002;Pierpoint et al., 1977). This kind of reaction can also be hypothesized to occur between two amino acids and one dimer of a phenolic compound. In the structure of the phenolic dimer proposed by Namiki and colleagues (Namiki et al., 2001), several sites seem to be available for covalent interaction with amino acid residues. In the presence of PPO, additional protein cross-linking may arise from the enzymatic hydroxylation of tyrosine into dihydroxyphenylalanine (DOPA), and its subsequent oxidation into a quinone. This quinone then reacts with another tyrosine (Aeschbach et al., 1976) or with other amino acid residues (Lissitzky et al., 1962). This modification of tyrosine by PPO can also explain the PPO induced formation of dimers of α-lactalbumin in the absence of CQA. The dimerisation of α-lactalbumin probably involves the modification of tyrosine residue 18 by PPO, as this tyrosine residue is the most accessible (http://ebi.ac.uk/thornton-srv/databases). The same phenomenon was observed when the peroxidase-induced dimerisation of α-lactalbumin in the presence of ferulic acid was studied (Oudgenoeg, 2004). With lysozyme, PPO did not induce a dimerisation in the absence of CQA, which may be related to the fact that the three tyrosine residues (residues 20, 23 and 53) of lysozyme seem not to be easily accessible for PPO (http://ebi.ac.uk/thornton-srv/databases).

In conclusion, modification of proteins by quinones at pH 9.0 (ALK), and 6.0 (PPO), 7.0 and 4.0 (via periodate, results not shown) had similar effects on the protein properties. This indicates that at neutral pH and mildly acidic pH, the same mechanism of modification occurs as at alkaline pH. In contrast to non-covalent interactions (**Chapter 2**), covalent interactions with CQA strongly modify the functional properties of proteins, such as

decreasing their solubility. The extent of modification and the effects of covalent modifications on protein functional properties may differ from protein to protein.

LITERATURE CITED

Aeschbach, R.; Amadò, R.; Neukom, H. Formation of dityrosine cross-links in proteins by oxidation of tyrosine residues. *Biochim. Biophys. Acta* **1976**, *439*, 292-301.

Caessens, P. W. J. R.; Gruppen, H.; Visser, S.; Van Aken, G. A.; Voragen, A. G. J. Plasmin hydrolysis of β-casein: foaming and emulsifying properties of the fractionated hydrolysate. *J. Agric. Food Chem.* **1997**, *45*, 2935-2941.

Charlton, A. J.; Baxter, N. J.; Lokman, M. K.; Moir, A. J. G.; Haslam, E.; Davies, A. P.; Williamson, M. P. Polyphenol/peptide binding and precipitation. *J. Agric. Food Chem.* **2002**, *50*, 1593-1601.

Cheynier, V.; Osse, C.; Rigaud, J. Oxidation of grape juice phenolic compounds in model solutions. *J. Food Sci.* **1988**, *53*, 1729-1732.

Church, F. C.; Swaisgood, H. E.; Porter, D. H.; Catignani, G. L. Spectrophotometric assay using O-phtaldialdehyde for determination of proteolysis in milk and isolated milk proteins. *J. Dairy Sci* **1983**, *66*, 1219-1227.

Felton, G. W.; Broadway, R. M.; Duffey, S. S. Inactivation of protease inhibitor activity by plant-derived quinones: complications for host-plant resistance against noctuid herbivores. *J. Insect Physiol.* **1989**, *35*, 981-990.

Fujita, Y.; Hidaka, Y.; Noda, Y. Thermal stability of alkylated and hydroxyalkylated lysozymes. *Thermochim. Acta* **1995**, *253*, 117-125.

García, P.; Concepción, R.; Brenes, M.; Garrido, A. Effect of metal cations on the chemical oxidation of olive *o*-diphenols in model systems. *J. Agric. Food Chem.* **1996**, *44*, 2101-2105.

Harrison, C. R.; Hodge, P. Polymer-supported periodate and iodate as oxidizing agents. *J. Chem. Soc. [Perkin. 1]*. **1982**, 509-511.

Hurrell, R. F.; Finot, P. A.; Cuq, J. L. Protein-polyphenol reactions. 1. Nutritional and metabolic consequences of the reaction between oxidized caffeic acid and the lysine residues of casein. *Br. J. Nutr.* **1982**, *47*, 191-211.

Kramer, K. J.; Kanost, M. R.; Hopkins, T. L.; Jiang, H.; Zhu, Y. C. Oxidative conjugation of catechols with proteins in insect skeletal systems. *Tetrahedron* **2001**, *57*, 385-392.

Kroll, J.; Rawel, H. M.; Rohn, S. Reactions of plant phenolics with food proteins and enzymes under special consideration of covalent bonds. *Food Sci. Technol. Res.* **2003**, *9*, 205-218.

Lissitzky, S.; Rolland, M.; Reynaud, J.; Lasry, S. Oxydation de la tyrosine et de peptides ou protéines la contenant par la polyphenoloxidase de champignon. III. Propriétés des protéines oxydées et de certaines DOPA-protéines. *Biochim. Biophys. Acta* **1962**, *65*, 481-494.

Namiki, M.; Yabuta, G.; Koizumi, Y.; Yano, M. Development of free radical products during the greening reaction of caffeic acid esters (or chlorogenic acid) and a primary amino compound. *Biosci. Biotechnol. Biochem.* **2001**, *65*, 2131-2136.

Oudgenoeg, G. Peroxidase catalysed conjugation of peptides, proteins and polysaccharides via endogenous and exogenous phenols. Ph.D. thesis, Wageningen, 2004.

Oudgenoeg, G.; Hilhorst, R.; Piersma, S. R.; Boeriu, C. G.; Gruppen, H.; Hessing, M.; Voragen, A. G. J.; Laane, C. Peroxidase-mediated cross-linking of a tyrosine-containing peptide with ferulic acid. *J. Agric. Food Chem.* **2001**, *49*, 2503-2510.

Peñalver, M. J.; Rodríguez-López, J. N.; García-Molina, F.; García-Cánovas, F.; Tudela, J. Method for the determination of molar absorptivities of thiol adducts formed from diphenolic substrates of polyphenol oxidase. *Anal. Biochem.* **2002**, *309*, 180-185.

Pierpoint, W. S. o-Quinones formed in plant extracts. Their reaction with bovine serum albumin. *Biochem. J.* **1969**, *112*, 619-629.

Pierpoint, W. S.; Ireland, R. J.; Carpenter, J. M. Modification of proteins during the oxidation of leaf phenols: reaction of potato virus X with chlorogenoquinone. *Phytochemistry* **1977**, *16*, 29-34.

Rawel, H. M.; Kroll, J.; Riese, B. Reactions of chlorogenic acid with lysozyme: physicochemical characterization and proteolytic digestion of the derivatives. *J. Food Sci.* **2000**, *65*, 1091-1098.

Rawel, H. M.; Kroll, J.; Rohn, S. Reactions of phenolic substances with lysozyme-physicochemical characterisation and proteolytic digestion of the derivatives. *Food Chemistry* **2001**, *72*, 59-71.

Rawel, H. M.; Rohn, S.; Kruse, H.-P.; Kroll, J. Structural changes induced in bovine serum albumin by covalent attachment of chlorogenic acid. *Food Chemistry* **2002**, *78*, 443-455.

Richard-Forget, F. C.; Gauillard, F. A. Oxidation of chlorogenic acid, catechins, and 4-methylcatechol in model solutions by combinations of pear (*Pyrus communis* Cv. Williams) polyphenol oxidase and peroxidase; a possible involvement of peroxidase in enzymatic browning. *J. Agric. Food Chem.* **1997**, *45*, 2472-2476.

Sarker, D. K.; Wilde, P. J.; Clark, D. C. Control of surfactant-induced destabilization of foams through polyphenol-mediated protein-protein interactions. *J. Agric. Food Chem.* **1995**, *43*, 295-300.

Siebert, K. J. Effect of protein-polyphenol interactions on beverage haze, stabilization, and analysis. *J. Agric. Food Chem.* **1999**, *47*, 353-362.

Singleton, V. L. Oxygen with phenols and related reactions in musts, wines and model systems: observations and practical implications. *Am. J. Enol. Vitic.* **1987**, *38*, 69-77.

Strauss, G.; Gibson, S. M. Plant phenolics as cross-linkers of gelatin gels and gelatin-based coacervates for use as food ingredients. *Food Hydrocolloids* **2004**, *18*, 81-89.

Chapter 4

Covalent interactions between oxidation products of caffeoylquinic acid and various amino acid side-chains

Chapter 4

Covalent interactions between oxidation products of caffeoylquinic acid and various amino acid side-chains

The covalent interactions between quinones from caffeoylquinic acid (CQA) and amino acid side-chains were studied with mass spectrometry using N-terminally protected amino acids. The addition of two molecules of CQA, presumably in the form of a pre-formed dimer, was observed for lysine, tyrosine, histidine and tryptophan. A monomer of CQA was able to react with histidine and tryptophan, whereas no interaction with a CQA monomer was observed for lysine and tyrosine. Serine and threonine showed no covalent interactions at all, neither with a monomer, nor with a dimer of CQA. Cross-linking between CQA and the side-chains of two molecules of lysine is likely to occur also in proteins. The results show that protein cross-linking may also be expected to occur via two tyrosine residues in the absence of other phenolic substrates. The side-chains of lysine and tyrosine are more reactive than that of histidine, while the reactivity of the side-chain of tryptophan is relatively low. These results show that covalent protein modification by oxidised phenolics occurs preferentially via an initial dimerisation and encompasses not only lysine and cysteine residues.

KEYWORDS: phenolic compounds; polyphenol oxidase; chlorogenic acid; LC-MS

*This chapter will be submitted for publication

1. INTRODUCTION

Physico-chemical properties of proteins, which are important in food industry, such as solubility, can be modified by covalent interactions of these proteins with oxidised phenolic compounds, i.e. quinones (**Chapter 3**;Rawel et al., 2001). Additionally, the nutritional value of proteins may be affected due to the modification of essential amino acids and through the inhibition of proteases (Kroll et al., 2003), a phenomenon used by plants as a defence mechanism against pathogens (Felton et al., 1989). Determining which amino acid side-chains can react with quinones, the oxidation products of phenolic compounds, is thus of particular importance in order to be able to modulate such protein-quinone interactions.

Upon modification of bovine serum albumin and soy proteins with caffeoylquinic acid (CQA, chlorogenic acid) and casein with caffeic acid, a decrease in the number of unmodified lysine, cysteine and tryptophan residues has been observed (Hurrell et al., 1982;Kroll et al., 2001;Rawel et al., 2002). However, these studies were focused on estimating the amount of the unmodified form of certain amino acids (lysine, tryptophan and cysteine residues), whereas the possible reactivity of other amino acids and the underlying mechanisms have not been investigated. In parallel with the characterisation of modified proteins, the reactions between quinones and free amino acids have been studied. The side-chain of histidine was shown to react with oxidised catechol and in this way to contribute to the sclerotization of the cuticle of insects (Xu et al., 1996). The side-chain of methionine seems also able to react with *o*-benzoquinone (Vithayathil and Satyanarayana Murthy, 1972). Cysteine was shown to covalently react with oxidised CQA (Pierpoint, 1966;Richard et al., 1991) and with other oxidised catechols (Peñalver et al., 2002). Using butylamine, an analogue for the side chain of lysine, and ethyl-caffeate, it was proposed that an initial dimerisation of the esters of caffeic acid takes place is followed by reaction of the thus formed dimer with primary amino compounds (Namiki et al., 2001;Yabuta et al., 2001). This phenolic dimerisation occurs via the binding of the isoprenyl group of the side-chain of two molecules of ethyl caffeate, which is followed by a cyclisation step and subsequently by a Michael addition of the amino compound (Namiki et al., 2001). Finally a nucleophilic cyclisation occurs with a concomitant loss of one water molecule (Namiki et al., 2001).

To be able to modulate the interactions between proteins and phenolic compounds, an inventory of amino acid side-chains susceptible to reaction with quinones is necessary. CQA was chosen for this study, as it is one of the most abundant simple phenolic compounds in

foods. In the previous chapter, it was already shown that the extent of protein modification by CQA cannot be ascribed solely to the modification of lysine and cysteine residues (**Chapter 3**). Prior to studying the reactivity of amino acids and the attachment of dimeric or monomeric CQA to the former, a pre-selection of amino acid residues was done. Based on the expected reactivity of their side-chains, the amino group of lysine, the pyrrole ring of tryptophan, the imidazole ring of histidine, the aromatic hydroxyl of tyrosine, the sulfur atom of methionine and the hydroxyl groups of serine and threonine were investigated in detail. Arginine, asparagine and glutamine were also tested for their reactivity, but were not expected to react easily. Cysteine was not studied in this paper as a product of CQA reaction with cysteine residues had already been characterised (Richard et al., 1991).
The characterisation of the adducts was performed using liquid chromatography - mass spectrometry.

2. MATERIAL AND METHODS

2.1 Materials

Caffeoylquinic acid (CQA; 1',3',4',5'-tetrahydroxycyclohexanecarboxylic acid 3-(3,4)-dihydroxypropenyldihydroxyphenyl-1-propenoate), amino acids and the corresponding amino acids protected at their N-terminal group by a tert-butyloxycarbonyl group (Boc) were purchased from Sigma Chemical Company (St Louis, MO, USA) and used without further purification. Tyrosinase from mushroom (EC 1.14.18.1, mPPO) was purchased from Fluka (Steinheim, Germany) and had an activity of 3216 U per mg (1 U oxidizing 1 μmol of 4-methylcatechol per min at pH 6.5 and 25^{o}C, Fluka).

2.2 Amino acid modification

Protected or unprotected amino acids (112 mM) were mixed with CQA (28 mM) and PPO (15 U/ml) in 0.1 M potassium phosphate buffer (pH 7.0). The molar ratio used during PPO oxidation was different from that used during alkaline conditions, because preliminary experiments with PPO showed that this molar ratio (4 moles of CQA per mole of amino acid) results in a visibly higher reaction rate than at lower and higher ratios. The pH of the mixtures was adjusted to pH 7.0 with 6N NaOH. Next, the samples were stirred for 24 h at 40 C° in a waterbath in open vials. Blanks of CQA, protected amino acid and non-protected amino acids were prepared. All samples were directly analysed by LC-MS after similar reaction times.

As PPO can hydroxylate (Boc-)tyrosine, the interactions were also studied using CQA oxidised at alkaline pH in order to be able to identify which products from the PPO mixture were not due to hydroxylation of tyrosine. Boc-tyrosine or tyrosine (28 mM) was mixed with CQA (28 mM) in water and the pH was adjusted to pH 9.0 with 6N NaOH. Next, the samples were stirred for 24h in open vials. Blanks consisting of CQA, Boc-tyrosine and tyrosine were prepared in a similar way. After dilution by a factor of 100, absorption spectra were recorded from 1100 nm to 190 nm using a Shimadzu UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan).

2.3 Liquid chromatography mass spectrometry (LC-MS)

Ten microliters of sample were applied to a Vydac C18 reversed-phase column (300 Å; 5 μm; 0.46 x 25 cm; Dionex, Sunnyvale, CA, USA) on a Spectra System HPLC (Thermo Separation Products, Inc., San Jose, CA, USA) coupled to a LCQ mass spectrometer (Finnigan MAT, San Jose, CA, USA). The solvents used were 0.15% (v/v) trifluoroacetic acid in water (solvent A) and acetonitrile (solvent B). The following gradient was applied: 0% B for 5 min, linear to 25% B between 5 and 45 min, linear to 50 % B between 45 and 68 min, linear to 100 % B between 68 and 77 min, 100% B between 77 min and 82 min, followed by reconditioning of the column at 0% B for 27 min. A flow rate of 0.2 ml/min was used. The absorbance of the eluate was monitored at 700 nm. The mass spectrum was collected using the positive electrospray ionization (ESI) mode with a capillary heated at 200 °C, a source voltage of 4.5 kV, a sheath gas flow of 60 arbitrary units with helium as the spraying gas. Full scan spectra were acquired from 100 to 2000 m/z. The tune method was made with a glycine tripeptide. MS-MS fragmentation patterns were also recorded. Data were processed using Xcalibur™ software (Finnigan MAT, San Jose, CA, USA).

3. RESULTS

3.1 Reaction products of oxidised CQA

Prior to the investigation of covalent products formed from oxidised CQA and amino acids, the molecular mass of the reaction products formed from CQA in the absence of amino acids was investigated. According to the mechanisms proposed for caffeic acid esters (Namiki et al., 2001;Yabuta et al., 2001) and caffeic acid (Cilliers and Singleton, 1991), formation of a

dimer of phenolic compounds can be expected upon oxidation (**Figure 1A**). Such a dimer of CQA should have a mass of 706.2 Da and this mass has been observed to encompass several dimeric structures (Antolovich et al., 2004;Bernillon et al., 2004b). The structure of a CQA dimer has been elucidated and was shown to correspond to the structure "b" presented in **Figure 1A**, having a mass of 706 Da (Bernillon et al., 2004a). The RP-HPLC total ion count (TIC) chromatogram (data not shown) was, therefore, screened for the presence of this mass. A compound with a m/z-value of 706.8, corresponding to 705.8 Da if a MH^+-ion is assumed, was indeed detected (**Figure 1B**). This m/z-value was detected at several retention times, which all gave the same MS/MS fragmentation spectra, indicating that several isomers of the 705.8 Da compound were presumably present. In addition to this compound, a m/z value of 532.8 was detected, which may correspond to the m/z-value of the dimer described previously without one quinic acid moiety (174 Da) (**Figure 1B**).

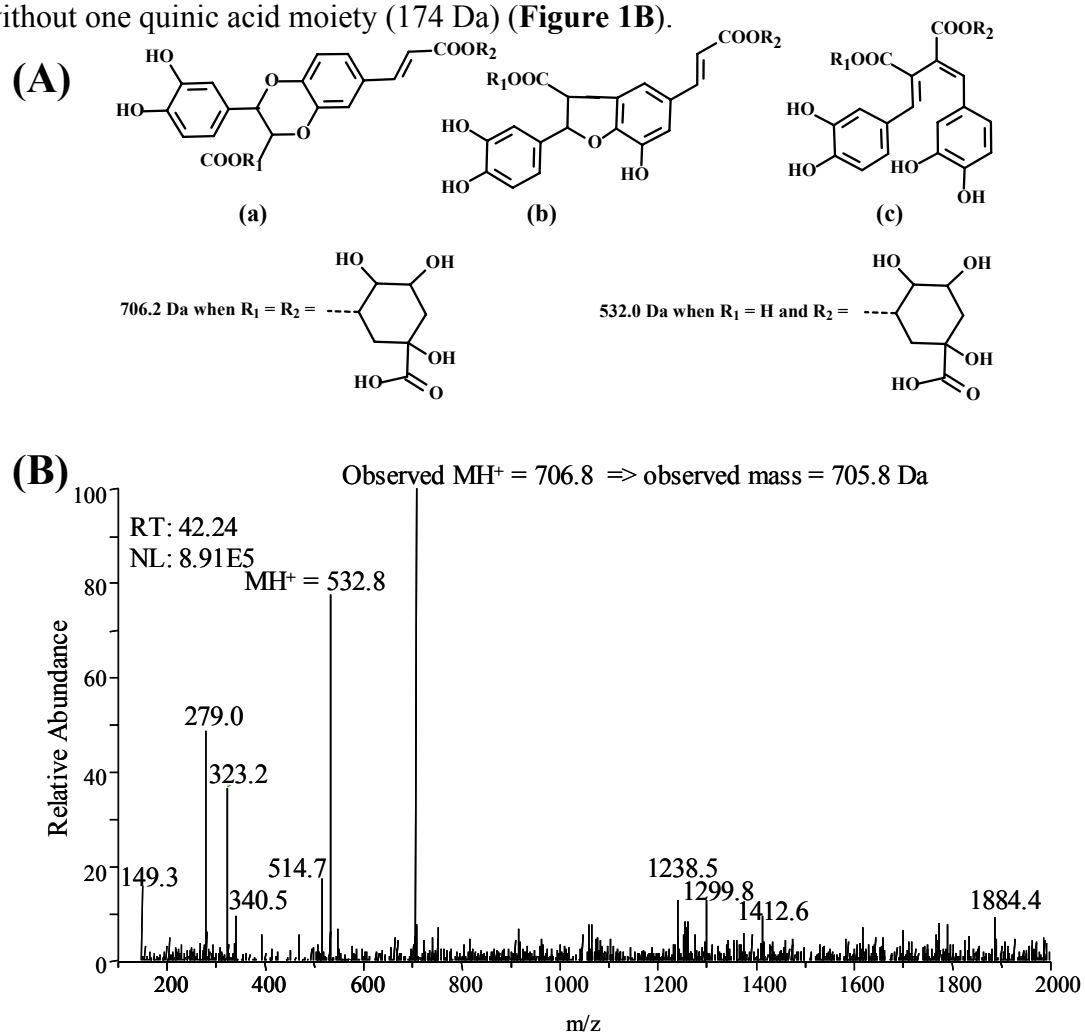


Figure 1. CQA incubated with PPO; A: hypothetical chemical structures (706.2 Da) according to the mechanisms observed for caffeic acid esters (Cilliers and Singleton, 1991;Namiki et al., 2001;Yabuta et al., 2001); B: Electrospray Ionisation (ESI) MS spectrum at a retention time (RT) of 42.24 min.

To determine whether CQA could react to multimers larger than dimers, the presence of CQA oligomers was investigated. The spectra were analysed up to 2000 m/z, but no peak with a significant intensity (intensity higher than 10^4-10^5), that could correspond to a CQA trimer or CQA tetramer, was detected.

3.2 Reaction products from oxidised CQA with amino acids

Incubation of oxidised CQA with unprotected amino acid resulted in the formation of a green colour. Since a green pigment has been observed upon reaction of primary amino groups with a dimer of oxidised ethyl caffeate (Namiki et al., 2001;Yabuta et al., 2001), this colour may indicate attachment of CQA dimers to the α-NH_2 group. Eleven amino acid residues protected at their N-terminal group were tested for their reactivity towards CQA: cysteine, lysine, arginine, asparagine, glutamine, tryptophan, histidine, tyrosine, methionine, serine and threonine. Reaction of oxidised CQA with these amino acids protected at their α-NH_2 group was investigated in the present study, with the exception of cysteine, as its reaction with oxidised CQA has already been investigated (Pierpoint, 1966;Richard et al., 1991). Of the ten amino acids studied, four resulted in the formation of reaction products (**Table 1**) and are, therefore, discussed further. The mass spectra of arginine, asparagine and glutamine only showed a low intensity of detection of the unmodified amino acids. No reaction products with these amino acids were detected in the spectra, indicating that arginine, asparagine and glutamine have a low reactivity towards CQA quinones, although the existence of reaction products could not be excluded.

Table 1. Tentative assignments of the m/z-values of reaction products between Boc-amino acids and PPO-oxidised CQA

mixture	mass(es) found	rel. intensity	hypothetical compound	theo. mass
Boc-Lys	929.1	high	Boc-Lys + CQA dimer	928.8
	825.3	high	Lys + CQA dimer	825.1
	1173.2	low	2 Boc-Lys + CQA dimer	1173.0
	1157.1	low	2 Boc-Lys + CQA dimer minus one hydroxyl group	1157.0
Boc-Trp	656.9	high	Boc-Trp + CQA monomer	657.2
	601.0	medium	Boc-Trp minus (C-$(CH_3)_3$) + CQA monomer	600.8
	1010.9	medium	Boc-Trp + CQA dimer	1011.0
Boc-His	962.0	low	Boc-His + CQA dimer	961.7
	610.1	low	Boc-His + CQA monomer	609.9
	785.9	low	Boc-His + CQA dimer without one quinic acid	785.9
Boc-Tyr	865.7	medium	Tyr + CQA dimer minus one hydroxyl group	865.9
	881.7	low	Tyr + CQA dimer	885.9
	985.7	medium	Boc-Tyr + CQA dimer	985.9
	1424.6/1426.6	medium		

The HPLC-MS spectra of protected amino acids incubated with oxidised CQA were screened for the presence of masses (m/z-values) that would indicate the presence of reaction products between CQA monomers or dimers and protected amino acids. The masses (m/z-values) detected in the protected amino acid blanks and in the reaction mixture of oxidised CQA with protected amino acids are presented in **Table 1**. In the mixtures of oxidised CQA with Boc-lysine, Boc-tryptophan, Boc-histidine and Boc-tyrosine, products, originating from the reaction of two molecules of oxidised CQA (likely a dimer of CQA), with the Boc-amino acids were detected (**Table 1**): a product of a high intensity and a m/z-value of 929.1 was formed from Boc-lysine (**Figure 2**), while a product with a medium intensity and a m/z-value of 1010.9 was present in modified Boc-tryptophan **(Figure 3**). The main product in modified Boc-histidine possessed a m/z-value of 962.0 (**Figure 4**), and a reaction product with a m/z-value of 985.7 was found in modified Boc-tyrosine (**Figure 5**).

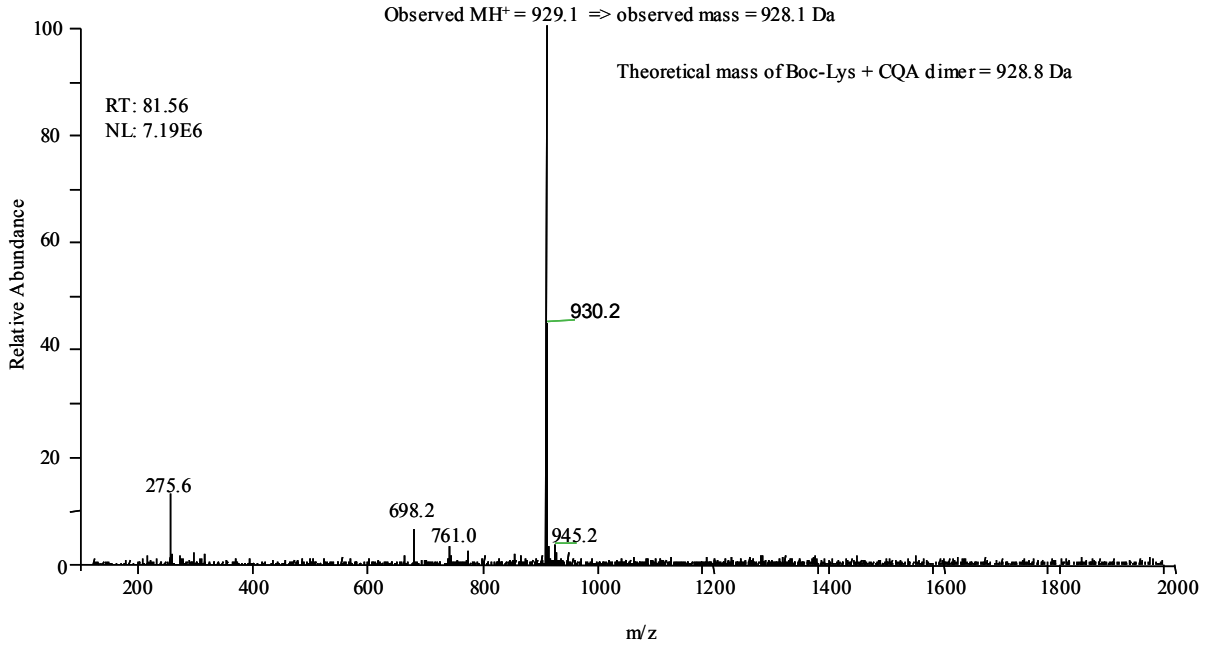


Figure 2. Electrospray Ionisation (ESI) MS spectrum of CQA modified Boc-lysine

In some Boc-amino acid-CQA mixtures, products originating from the reaction between a single CQA molecule and the Boc-amino acid were present (**Table 1**). The main product in modified Boc-tryptophan possessed a m/z-value of 656.9 (**Figure 3**), while a compound with a m/z-value of 610.1 was detected in the Boc-histidine-CQA mixture (**Figure 4**).

Because PPO is able to hydroxylate and subsequently oxidise Boc-tyrosine into a quinone, the covalent reaction between CQA and Boc-tyrosine was also studied in the

absence of PPO, using CQA oxidised at alkaline pH. The same m/z-values were observed from the TIC chromatograms of the incubation mixture of Boc-tyrosine and CQA in the presence of PPO as at alkaline pH (**Table 1**).

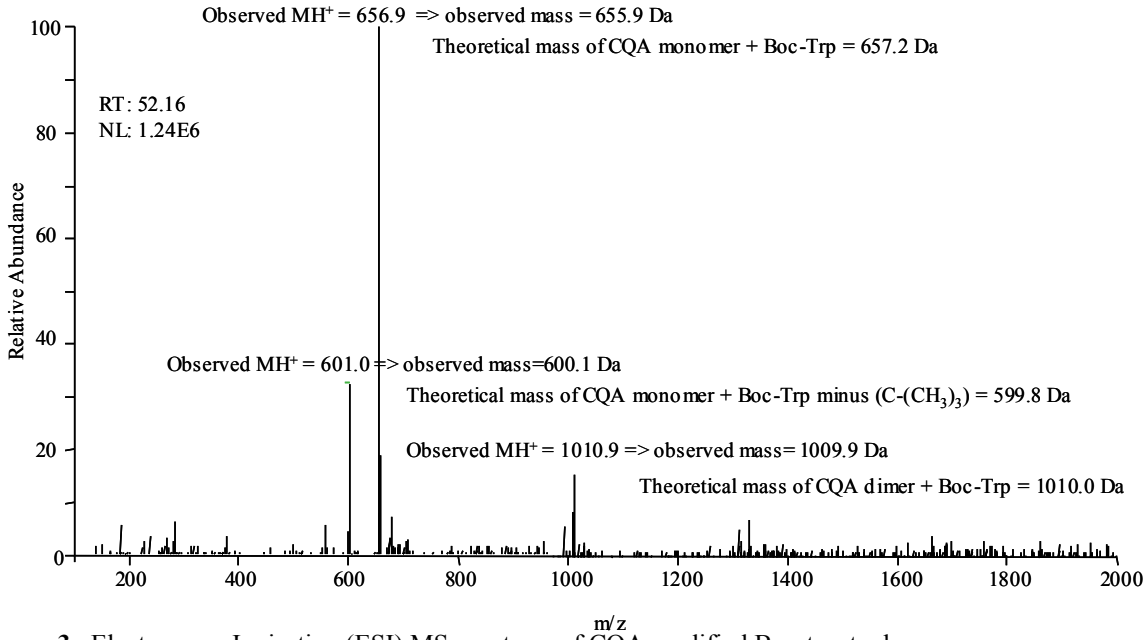


Figure 3. Electrospray Ionisation (ESI) MS spectrum of CQA modified Boc-tryptophan

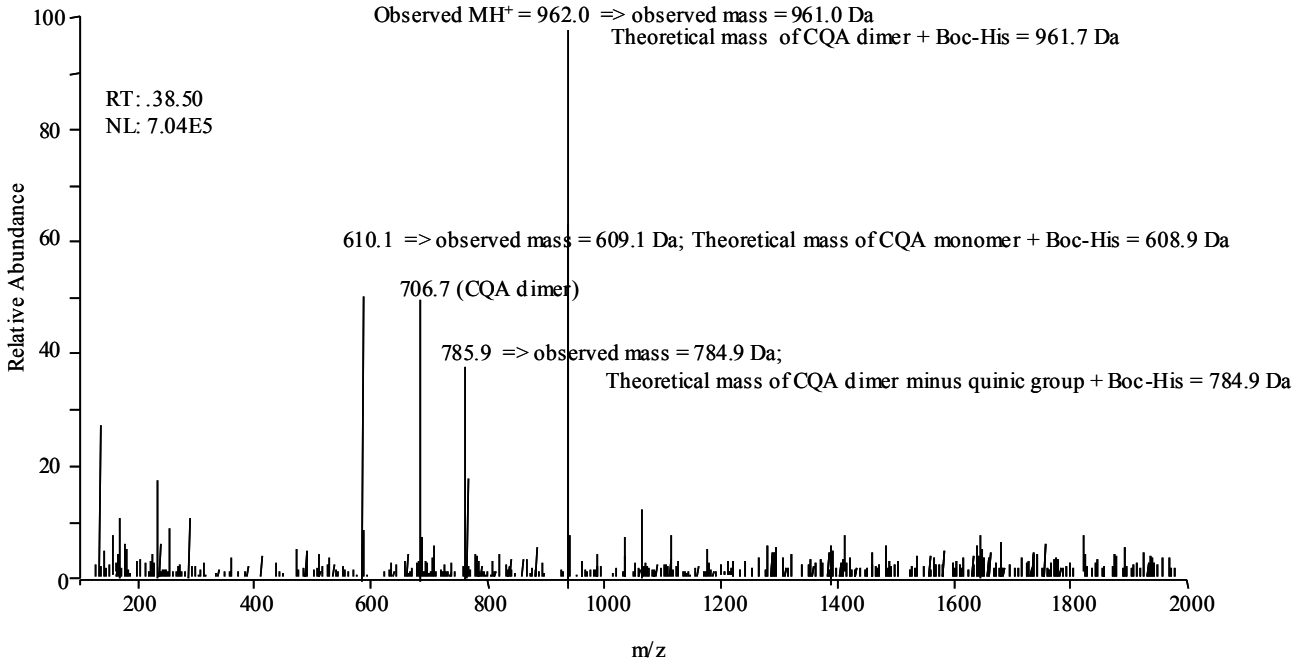


Figure 4. Electrospray Ionisation (ESI) MS spectrum of CQA modified Boc-histidine

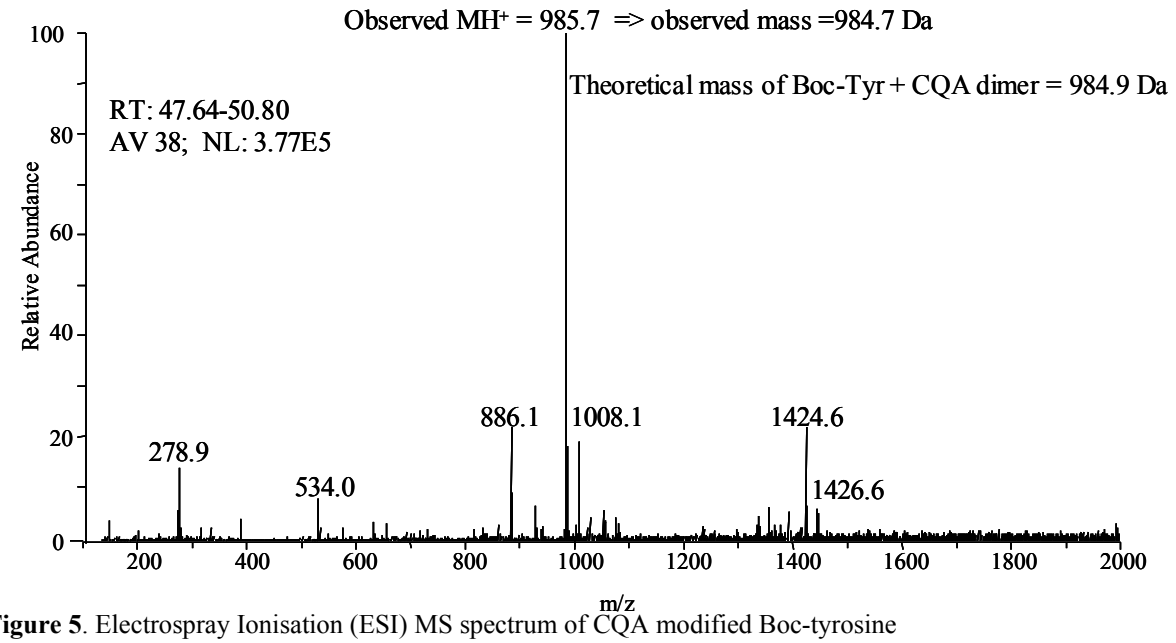


Figure 5. Electrospray Ionisation (ESI) MS spectrum of CQA modified Boc-tyrosine

In the TIC-chromatograms of the incubation mixture of Boc-lysine and CQA, compounds with m/z values of 1157.1 and 1173.2 were detected at retention times of 93 and 97 min, with low intensities (data not shown). These compounds can be expected to derive from the reaction of two molecules of Boc-lysine with dimeric oxidised CQA (**Table 1**). No reaction products of CQA with lysine protected at both α– and ε–NH_2 groups were detected, confirming that the reaction site of CQA on Boc-lysine is indeed the ε– NH_2 group.

In some cases, cleavage of the whole Boc-group or of the (C-$(CH_3)_3$) part of the Boc group was observed in the blank and/or the mixture with CQA, leading to a loss of 100 Da or 56 Da, respectively (**Table 1**).

4. DISCUSSION

4.1 Reaction mechanisms

In all incubation mixtures, CQA dimers were observed and were expected to be the main compound reacting with amino acids, as was also observed for caffeic acid esters by Namiki and co-workers (Namiki et al., 2001). Indeed, MS analyses indicated that reaction products between an oxidised CQA dimer and Boc-amino acids were observed for Boc-lysine, Boc-tryptophan, Boc-histidine and Boc-tyrosine. However, a monomer of oxidised CQA was shown to be able to react with tryptophan and histidine, although no reaction between a CQA-

monomer and lysine or tyrosine was observed. The higher apparent reactivity of the oxidised CQA dimer compared to the oxidised CQA monomer, observed with lysine and tyrosine, in this paper and in the previous chapter describing protein modification (**Chapter 3**), could result from the lower redox potential of phenolic dimers compared to monomers (Singleton, 1987). An addition of 682 Da was observed for Boc-lysine, whereas the molecular mass of a CQA dimer observed in the absence of amino acid was 706 Da. This difference can be explained by the loss of one hydroxyl group and the occurrence of several oxidation steps after binding of the CQA dimer to Boc-lysine. Such a rearrangement has been proposed for the reaction of caffeic acid with butylamine and results in incorporation of the amino nitrogen in a ring structure (Namiki et al., 2001), as indicated in **Figure 6A**. The final product shown in **Figure 6A** corresponds to lysine that is modified by a mass addition of a 682 Da moeity. Since, a mass addition of 706 Da was observed for Boc-tryptophan and Boc-histidine, and a mass addition of 704 Da was observed for Boc-tyrosine, such a rearrangement probably does not occur with tryptophan, histidine and tyrosine. This can be explained by the fact that, in contrast to the ε-NH_2 group of lysine, the nitrogen atom of histidine and the oxygen atom of tyrosine side-chain can bind to only one other atom, making such a ring closure impossible**.** The same applies for tryptophan, in which a carbon atom, rather than the nitrogen atom, of the pyrrole ring, is expected to react with the quinone, as observed for the reaction between substituted pyrroles and 1,4-benzoquinone (Finley, 1974). The structure of the hypothetical compounds that could result from covalent modification of tyrosine, tryptophan and histidine after reaction with a CQA dimer are presented in **Figure 6B**. Since mainly mass additions corresponding to rearrangements similar to those observed with lysine are observed for CQA modified proteins (**Chapter 3**), lysine residues in proteins seem to be the most susceptible for CQA modification.

No oligomers higher than a dimer were detected, indicating that CQA hardly forms multimers higher than dimers. This is an indication that additions of more than two CQA molecules per protein molecule, as observed with protein modification studies (**Chapter 3**; Rawel et al., 2000), are the result of the binding of several dimers (or monomers) to several protein sites rather than of the binding of CQA oligomers.

A compound, with a m/z-value of 1173.2, observed during Boc-lysine modification may have been formed from a dimer of CQA that has reacted with two molecules of Boc-lysine. Such a reaction could be responsible for the observed cross-linking of amino acid residues in proteins (**Chapter 3**). Several other reactions responsible for cross-linking can be expected, especially

in the case of tyrosine enzymatically modified into dihydroxyphenylalanine (Haemers et al., 2003).

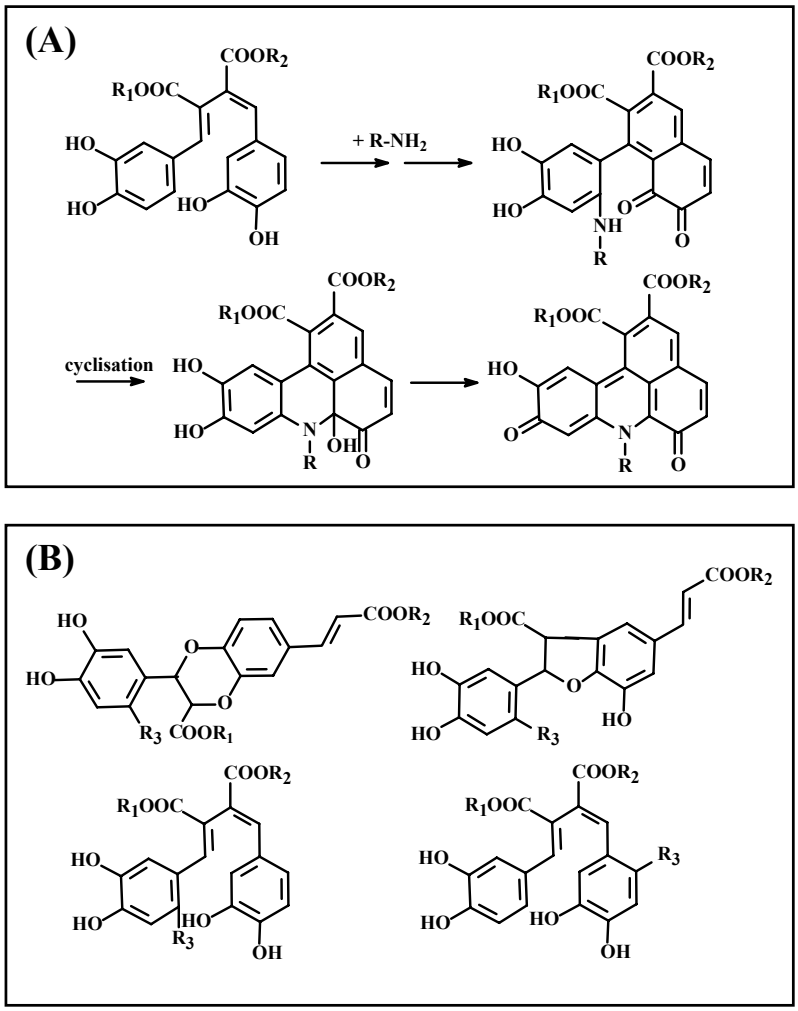


Figure 6. Proposed structures of reaction products formed from amino acid side-chains and CQA dimers. A: mechanism and structure of lysine bound to a CQA dimer, according to Namiki and co-workers (Namiki et al., 2001); B: structure of tyrosine, histidine and tryptophan bound to a CQA dimer, according to the structure of phenolic dimers (Cilliers and Singleton, 1991;Namiki et al., 2001). R_1 and R_2: quinic acid moiety (see Figure 1); R-NH_2: lysine residue; and R_3: amino acid side-chain.

Despite the possible hydroxylation and subsequent oxydation of Boc-tyrosine by PPO, no additional reaction products between Boc-tyrosine and CQA were observed in the presence of PPO compared to those formed at alkaline pH. This can be explained by the fact that PPO

has usually a higher affinity for o-diphenols, such as CQA, than for monophenols such as tyrosine (García-Molina et al., 2005).

4.2 Comparison of the reactivity of amino acids

The purpose of this paper was to determine which amino acid residues are susceptible to react with oxidised CQA, in order to control the interactions of CQA with proteins. An absolute comparison of the reactivity of the different Boc-amino acids is difficult, because no quantification of the reaction products could be performed. By comparing the intensities of the RP-HPLC-MS spectra of the Boc-amino acids with those of unreacted Boc-amino acids, it seems that about 75% of the available Boc-lysine and Boc-tyrosine have reacted, whereas about 30% of the Boc-histidine and only 1-5% of the Boc-tryptophan has reacted. Therefore, lysine and tyrosine may be also expected to be the most susceptible residues to react with oxidised CQA in proteins, whereas methionine, serine, threonine, arginine, asparagine and glutamine do not seem to react.

LITERATURE CITED

Antolovich, M.; Bedgood, D. R. J.; Bishop, A. G.; Jardine, D.; Prenzler, P. D.; Robards, K. LC-MS investigation of oxidation products of phenolic antioxidants. *J. Agric. Food Chem.* **2004**, *52*, 962-971.

Bernillon, S.; Personal communication **2005**.

Bernillon, S.; Renard, M. G. C.; Guyot, S. An LC/MS approach to study oxidation products of polyphenolic compounds in cider apple juice. *XXII International Conference on Polyphenols, Helsinki, Finland*; 2004, Hoikkala, A.; Soidinsalo, O., Ed.; Gummerus Printing, p 557-558.

Cilliers, J. J. L.; Singleton, V. L. Characterization of the products of nonenzymic autoxidative phenolic reactions in a caffeic acid model system. *J. Agric. Food Chem.* **1991**, *39*, 1298-1303.

Felton, G. W.; Broadway, R. M.; Duffey, S. S. Inactivation of protease inhibitor activity by plant-derived quinones: complications for host-plant resistance against noctuid herbivores. *J. Insect Physiol.* **1989**, *35*, 981-990.

Finley, K. T. The addition and substitution chemistry of quinones. In *The chemistry of functional groups. The chemistry of the quinonoid compounds*; S. Patai, Ed.; Interscience Publication: London, 1974; pp 877-1144.

García-Molina, F.; Peñalver, M. J.; Fenoll, L. G.; Rodríguez-López, J. N.; Varón, R.; García-Cánovas, F.; Tudela, J. Kinetic study of monophenol and *o*-diphenol binding to *oxy*tyrosinase. *J. Mol. Catal.* **2005**, *32*, 185-192.

Haemers, S.; Koper, G. J. M.; Frens, G. Effect of oxidation rate on cross-linking of mussel adhesive proteins. *Biomacromolecules* **2003**, *4*, 632-640.

Hurrell, R. F.; Finot, P. A.; Cuq, J. L. Protein-polyphenol reactions. 1. Nutritional and metabolic consequences of the reaction between oxidized caffeic acid and the lysine residues of casein. *Br. J. Nutr.* **1982**, *47*, 191-211.

Kroll, J.; Rawel, H. M.; Rohn, S. Reactions of plant phenolics with food proteins and enzymes under special consideration of covalent bonds. *Food Sci. Technol. Res.* **2003**, *9*, 205-218.

Kroll, J.; Rawel, H. M.; Rohn, S.; Czajka, D. Interactions of glycinin with plant phenols - influence on chemical properties and proteolytic degradation of the proteins. *Nahrung/Food* **2001**, *45*, 388-389.

Namiki, M.; Yabuta, G.; Koizumi, Y.; Yano, M. Development of free radical products during the greening reaction of caffeic acid esters (or chlorogenic acid) and a primary amino compound. *Biosci. Biotechnol. Biochem.* **2001**, *65*, 2131-2136.

Peñalver, M. J.; Rodríguez-López, J. N.; García-Molina, F.; García-Cánovas, F.; Tudela, J. Method for the determination of molar absorptivities of thiol adducts formed from diphenolic substrates of polyphenol oxidase. *Anal. Biochem.* **2002**, *309*, 180-185.

Pierpoint, W. S. The enzymic oxidation of chlorogenic acid and some reactions of the quinone produced. *Biochem. J.* **1966**, *98*, 567-580.

Rawel, H. M.; Kroll, J.; Hohl, U. C. Model studies on reactions of plant phenols with whey proteins. *Nahrung* **2001**, *45*, 72-81.

Rawel, H. M.; Kroll, J.; Riese, B. Reactions of chlorogenic acid with lysozyme: physicochemical characterization and proteolytic digestion of the derivatives. *J. Food Sci.* **2000**, *65*, 1091-1098.

Rawel, H. M.; Rohn, S.; Kruse, H.-P.; Kroll, J. Structural changes induced in bovine serum albumin by covalent attachment of chlorogenic acid. *Food Chemistry* **2002**, *78*, 443-455.

Richard, F. C.; Goupy, P. M.; Nicolas, J. J.; Lacombe, J.-M.; Pavia, A. A. Cysteine as an inhibitor of enzymatic browning.1. Isolation and characterization of addition compounds formed during oxidation of phenolics by apple polyphenol oxidase. *J. Agric. Food Chem.* **1991**, *39*, 841-847.

Singleton, V. L. Oxygen with phenols and related reactions in musts, wines and model systems: observations and practical implications. *Am. J. Enol. Vitic.* **1987**, *38*, 69-77.

Vithayathil, P. J.; Satyanarayana Murthy, G. New reaction of *o*-benzoquinone at the thioether group of methionine. *Nature. New Biol.* **1972**, *236*, 101-103.

Xu, R.; Huang, X.; Morgan, T. D.; Prakash, O.; Kramer, K. J.; Hawley, M. D. Characterization of products from the reactions of *N*-acetyldopamine quinone with *N*-acetylhistidine. *Arch. Biochem. Biophys.* **1996**, *329*, 56-64.

Yabuta, G.; Koizumi, Y.; Namiki, K.; Hida, M.; Namiki, M. Structure of green pigment formed by the reaction of caffeic acid esters (or chlorogenic acid) with a primary amino compound. *Biosci. Biotechnol. Biochem.* **2001**, *65*, 2121-2130.

Chapter 5

Interactions between globular proteins and procyanidins of different degrees of polymerization

Chapter 5

Interactions between globular proteins and procyanidins of different degrees of polymerization

The affinity of globular proteins towards procyanidins of various degrees of polymerisation (DP) leading to non-covalent interactions was measured and their effects on protein solubility and foam properties were examined. Whereas epicatechin and procyanidin dimers weakly interacted, α-lactalbumin showed a medium to high affinity for procyanidins of an average DP of 5.5 and 7.4, respectively. A positive cooperativity of binding at low procyanidin-protein molar ratios was observed. The affinity of α-lactalbumin and lysozyme for procyanidins increased when the pH was close to the isoelectric pH. Addition of procyanidins of DP 5.5 strongly decreased the solubility of lysozyme and to a lesser extent those of α-lactalbumin and BSA. Increasing the ionic strength had no effect on protein solubility, whereas increasing the temperature slightly increased the protein solubility. Procyanidins of DP 5.5 and 7.4 stabilised the average bubble diameter of foam formed with α-lactalbumin, but had no effect on foam made from BSA. These results indicate that procyanidins of medium DP can lead to an undesirable decrease of protein solubility, but may play a positive role in foam stability.

KEYWORDS: Proanthocyanidins; polyphenols; tannins; isothermal titration calorimetry (ITC); air-water interface; hydrogen bonding

*This chapter will be submitted for publication

1. INTRODUCTION

Proanthocyanidins are oligomeric and polymeric phenolic compounds that are common in some cereals, some legume seeds, cacao, tea, fruits and grapes (Santos-Buelga and A., 2000). In foods, the most common proanthocyanidins are the procyanidins, which are composed of units of catechin and/or epicatechin, and the prodelphinidins, which are composed of units of gallocatechin and/or epigallocatechin (Santos-Buelga and A., 2000). In the last decade, proanthocyanidins have been intensively studied for their antioxidant properties (Hagerman et al., 1998b;Riedl and Hagerman, 2001).

The interactions of proanthocyanidins with proteins can modify the functional properties of food proteins, as these interactions often result in a decrease of protein solubility (Hagerman and Butler, 1980;Kumar and Horigome, 1986). The pH of maximum protein precipitation was reported to be close to or below the isoelectric point of the proteins (Hagerman and Butler, 1981;Naczk et al., 1996).

Proanthocyanidins are believed to mainly bind to proteins via non-covalent interactions. But covalent interactions may also occur. Covalent interactions may take place after degradation of proanthocyanidins at low pH (Swain and Hillis, 1959;Torres and Bobet, 2001) or, like other phenols, may occur after their oxidation into quinones (Pierpoint, 1969). The non-covalent interactions between proanthocyanidins and proteins have been studied more extensively than the covalent interactions, but the precise nature of the former interactions still remains largely unclear. Both hydrophobic interactions and hydrogen bonding have been suggested (Hagerman et al., 1998a;Haslam, 1989). Using a protein precipitation assay, procyanidins have been reported to interact with bovine serum albumin (BSA) via hydrogen bonds rather than via hydrophobic interactions (Hagerman et al., 1998a). In contrast to these results, procyanidins were reported to mainly interact with BSA via hydrophobic interactions, as concluded from ultrafiltration measurements (Artz et al., 1987).

The protein precipitating effectiveness of mixtures of proanthocyanidins and proanthocyanidin gallates seems to increase with increasing degrees of polymerisation (DP), until an optimal DP of 12 units of catechin (De Freitas and Mateus, 2001a). Above this DP, the precipitating capacity decreases because of steric hindrance (De Freitas and Mateus, 2001a). Relatively open proteins, such as non-structured and proline-rich proteins, seem to have a higher affinity for proanthocyanidins than the more closed globular proteins (De Freitas and Mateus, 2001a;Hagerman and Butler, 1981). The interactions of

proanthocyanidins with proline-rich proteins have been extensively studied (Baxter et al., 1997;Charlton et al., 2002;De Freitas and Mateus, 2001b). The interactions with the, for food purposes more important, globular proteins have not been studied extensively. Understanding the nature and the extent of interactions between proanthocyanidins and globular proteins is necessary to be able to control and modulate the properties and behaviour of these proteins that are important in food industry.

Studies of protein interactions with proanthocyanidins including gallocatechins have been limited so far to the study of interactions with procyanidin monomers, dimers and trimers and mixtures of proanthocyanidins and proanthocyanidin gallates of various DP (Artz et al., 1987;Asquith and Butler, 1986;De Freitas and Mateus, 2001a;Hagerman and Butler, 1981;Kumar and Horigome, 1986;Naczk et al., 1996;Oh et al., 1980). These studies have been restricted to turbidimetry, protein precipitation and ultrafiltration measurements, which might be not suitable to measure the real affinity of proteins for proanthocyanidins. In this paper, the affinity between procyanidins of various degrees of polymerisation and α-lactalbumin and lysozyme at various pHs was measured using isothermal titration calorimetry. α-Lactalbumin, lysozyme and BSA were chosen as models for globular proteins in this study because α-lactalbumin and lysozyme have highly similar amino acid sequences, but a different pI and hydrophobicity. On the other hand, α-lactalbumin and BSA have similar pI values, but BSA possesses a higher molecular mass than α-lactalbumin. The comparisons of procyanidin interactions using three globular proteins, which possess similarities and differences, have not been investigated previously, apart from measuring their effects on protein precipitation capacity (Hagerman and Butler, 1981). In the present study, the effects on protein solubility as a function of pH are studied at various temperatures and ionic strengths. The effects of procyanidin interactions on foam properties of proteins were also investigated.

2. MATERIAL AND METHODS

2.1 Materials

Bovine α-lactalbumin (type I, holo-α-lactalbumin), lysozyme (from chicken egg white), bovine serum albumin (BSA, fractionated by cold alcohol precipitation and essentially fatty acid free), and (-)-epicatechin were purchased from Sigma Chemical Company (St Louis, MO, USA) and used without further purification.

2.2 Purification of procyanidins

Procyanidin dimers and procyanidins of medium degrees of polymerisation (DP) were purified from 320 g of a methanolic extract of cider apples var. Jeanne Renard (JR). The procyanidins of a higher degree of polymerisation were purified from 8 g of an acetonic extract of cider apples var. Marie Ménard (MM). These fractions, prepared as described by Guyot and co-workers (Guyot et al., 2001), were further purified using solid phase extraction (Guyot et al., 2001). A total amount of 3.481 g of JR polyphenolic fractions and 1.435 g of MM polyphenolic fractions were obtained. The JR fractions were further purified using normal-phase HPLC (Guyot et al., 2001). From the eluates, a fraction enriched in dimers and several fractions of medium DP were collected. The fraction enriched in dimers, which represented a total of 450 mg, was further purified using reversed-phase HPLC (Guyot et al., 2001). The 0.7 g of MM polyphenolic fractions, which were obtained using solid phase extraction, were further purified on a Toyopearl TSK HW-40 (12 x 1 cm) column (Tosohaas, Japan) as described by Le Bourvellec and co-workers (Le Bourvellec et al., 2004). A quantity of 831 mg of procyanidins of the higher DP was obtained. The purity and the average DP of the different fractions were determined by thiolysis of the samples followed by HPLC analysis (Guyot et al., 2001).

2.3 Procyanidin composition

The fraction of purified dimers contained 91.5 % of procyanidin dimers, 6.0% of procyanidin oligomers, 1.8% of epicatechin, 0.6% of caffeoylquinic acid (chlorogenic acid) and < 0.2% of phloridzin (dihydrochalcones). The three fractions of medium DP contained only procyanidins (monomers, oligomers and polymers of procyanidins) and had average DPs of 5.0 and 5.5. The purified MM fraction (831 mg) contained only procyanidins and had an average DP of 7.4. As expected for extracts of cider apple (Escarpa and González, 1998;Sanoner et al., 1999), the procyanidin fractions mainly consisted of (-)-epicatechin units and the dimeric fraction mainly consisted of procyanidin B2 (epicatechin-(4β→8)-epicatechin).

2.4 Isothermal titration calorimetry (ITC)

The heat effects of successive additions of procyanidins to protein solutions were measured with a MCS isothermal titration calorimeter (MicroCal, Inc., Northampton, MA, USA). Solutions of procyanidins and proteins were degassed during 25 minutes under vacuum. The

reference cell contained degassed water. The sample cell (1.3 ml) contained protein (0.0047-0.0187 mM), while a solution of procyanidins (3.16 mM) was in the 250μL syringe. The initial delay was 600 seconds before the first injection, which consisted of 2 μL of procyanidin solution, was added to the protein solution in a span of 5.0 seconds. Every 800 seconds, the syringe injected 5 μL in a span of 12.6 seconds for the second and the third injections, and 10 μL in a span of 25.1 seconds for the fourth until the 26th injection. The delay between the injections was reduced to 500 seconds when the time to get back to equilibrium allowed it. The reference offset was 50 %. Samples with only protein or procyanidins were used as blanks, and the data of the sample containing only procyanidins were subtracted from data of protein with procyanidins. The heat changes were analysed with the use of the Origin software (MicroCal Software, Inc., Northampton, MA, USA). Experiments were performed at 25 ^{o}C.

Solutions of 3.16 mM procyanidins (DP5.5), α-lactalbumin and lysozyme were prepared in buffers with an ionic strength (I) of 0.023 M: 26 mM sodium phosphate buffer (pH 3.0), 27 mM sodium acetate buffer (pH 5.5) and 10 mM sodium phosphate buffer (pH 7.5). The protein concentrations were 0.0187 mM at pH 5.5 and 7.5, and 0.0047 mM at pH 3.0. The effect of the procyanidin DP on the interactions was studied at pH 5.5 using α-lactalbumin with (-)-epicatechin and procyanidin preparations with average DPs of 2, 5.5 and 7.4.

2.5 Protein content

The nitrogen content of the supernatant was measured using the Dumas combustion method on a NA 2100 nitrogen and protein analyzer (ThermoQuest, Rodano, Italy) according to the instructions of the manufacturer. After calibration with urea or methionine, the protein concentration was calculated using the nitrogen-protein conversion factors of 6.25 for α-lactalbumin, 5.29 for lysozyme and 6.02 for BSA.

2.6 Protein solubility

α-Lactalbumin [0.50% (w/v)] and DP5.5 procyanidins [0.29% (w/v)] were incubated at a ratio of 5 moles of procyanidins per mole of protein in 26.6 mM (I = 0.023 M), 50 mM (I = 0.043 M) or 100 mM (I = 0.087 M) sodium acetate buffer (pH 5.5), at 10^{o}C, room temperature or 40^{o}C. After 2h of incubation, aliquots (0.15 ml) were collected, immediately

centrifuged (1 min, 12,700 x g, 25°C) and the supernatants were analysed for their protein contents. A part of the samples incubated at room temperature was analysed after 72 h of incubation. Prior to incubation, one µl of 10% (w/v) sodium azide was added per ml of solution. The protein contents of the supernatants and of the non-centrifuged samples were determined using the Dumas combustion method, as described previously. The protein content of the samples incubated for 72h was corrected for the nitrogen due to the addition of azide. Blanks consisted of centrifuged and non-centrifuged samples of protein without procyanidins and procyanidins without protein. The protein content of the supernatant of the protein blank was set at 100% and defined as 100% solubility.

α-Lactalbumin, lysozyme and BSA [0.5% (w/v)], in the absence or presence of procyanidins of DP 7.4, were dissolved in 0.01 M sodium phosphate buffer (pH 7.5; I = 0.023 M). The protein solutions were subsequently adjusted to various pHs (pH 2.0 to pH 10.0) with NaOH or HCl (1 M or 6 M). After two hours, the samples were centrifuged (15 min, 12,700 x g, 25°C). Each pH was studied in triplicate. The protein contents of the samples were measured as described previously and were corrected for the dilution with NaOH or HCl.

2.7 Air-water interfacial and foam properties

The air-water interfacial and foam properties of α-lactalbumin and BSA were studied in the presence of procyanidins of DP 2.0, 5.0, and 7.4 at pH 7.0, and with procyanidins of an average DP of 5.0 at pH 4.0. The ratios used were 2 and 5 moles of procyanidins per mole of protein. Buffers used were 17 mM sodium phosphate buffer (pH 7.0; I = 30 mM) and 100 mM sodium acetate buffer containing 13 mM of sodium chloride (pH 4.0; I = 30 mM).

The surface tension of samples was determined using an automatic drop tensiometer (ADT- Tracker, I.T. Concept, Longessaigne, France). The shape of an air bubble in rising configuration in a protein solution [0.01% (w/v)] in the absence or presence of procyanidins was analysed digitally. After 3600 sec, the elastic modulus was obtained during dynamic oscillation of the area of the bubble with a relative area deformation of 6.56%.

Foam was prepared according to the whipping method of Caessens and co-workers (Caessens et al., 1997). A protein solution [1% (w/v)] in the absence or presence of procyanidins was whipped for 3 min at 2500 rpm for samples at pH 4.0 and at 3500 rpm for samples at pH 7.0. Next, the samples were poured into a cuvette (45 x 57 x 134 mm). The mean bubble diameter

(d_{21}) was determined from images of the cuvettes taken in reflection mode via a prism, every 60 seconds during 1h.

3. RESULTS

3.1 Effect of procyanidin size on protein-procyanidin interactions

Protein-procyanidin interactions were studied using ITC by measuring the heat released by procyanidin additions to solutions of α-lactalbumin and lysozyme. When procyanidins of average DPs of 5.5 and 7.4 were injected to a cell containing only buffer, a positive enthalpy change (ΔH_{obs}) was measured reaching a maximum of about 300 J/mole of procyanidins (data not shown). Since procyanidins are known to be able to aggregate with each other, presumably via hydrophobic interactions (Riou et al., 2002), such a positive (endothermic) enthalpy change upon dilution into the buffer-containing cell may indicate deaggregation of phenolic compounds, as also proposed for hydrolysable tannins by Frazier and co-workers (Frazier et al., 2003). Enthalpy changes due to protein-procyanidin interactions were corrected for this positive enthalpy. The titration curves showing the enthalpy changes upon titration of α-lactalbumin with epicatechin, procyanidin dimers and procyanidins of average DPs of 5.5 and 7.4, are presented in **Figure 1**. Epicatechin and the procyanidin dimer did not induce pronounced enthalpy changes (**Figure 1**). Procyanidins of an average DP of 5.5 and an average DP of 7.4 induced enthalpy changes, which occurred in two stages (**Figure 1**). In the first stage, i.e. at ratios ≤ 3-5 moles of procyanidins per mole of protein, the enthalpy change became more and more negative with every addition of procyanidins, indicating that more and more energy was released per injection of procyanidins (**Figure 1**). In the second stage of protein-procyanidin interactions, the exothermic effect decreased until reaching a plateau, indicating saturation of the protein (**Figure 1**). The more pronounced the slope, the higher the affinity. The binding affinities obtained between protein and procyanidins and the number of binding sites on the protein molecules are presented in **Table 1**. α-Lactalbumin exhibited a higher affinity for DP 7.4 procyanidins than for DP 5.5 procyanidins, whereas affinities for epicatechin and procyanidin dimers were too low to be quantified (**Table 1**). The affinities corresponding to the first stage of interactions, i.e. at low molar ratios, are not presented in **Table 1**, because too few data points were available to obtain a satisfactory curve fitting.

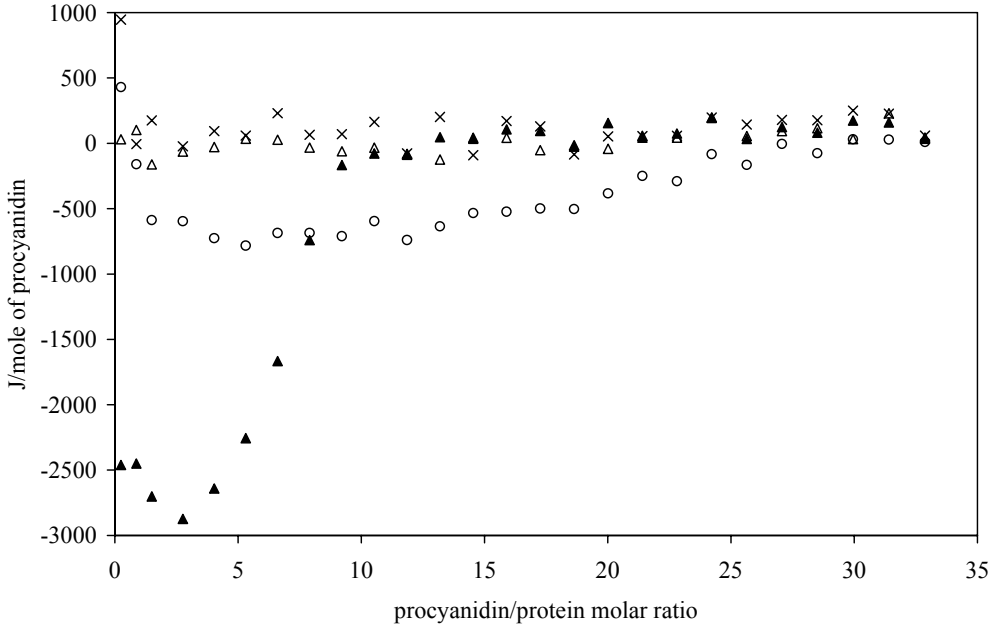


Figure 1. Isothermal titration calorimetry (25°C) of α-lactalbumin titrated with procyanidins of various DPs: (x) epicatechin; (△) procyanidin dimer; (○) procyanidins of DP 5.5; (▲) procyanidins of DP 7.4.

Table 1. Apparent binding affinity constant (K) and number of binding sites per protein molecule (N), of the second stage of protein-procyanidin interactions, at various pHs and with procyanidins of DP 1 (epicatechin), DP2, average DP of 5.5 and average DP of 7.4.

	lysozyme			**α-lactalbumin**					
	pH 3.0	**pH 5.5**	**pH 7.5**	**pH 3.0**	**pH 5.5**				**pH 7.5**
procyanidin DP	**5.5**	**5.5**	**5.5**	**5.5**	**1**	**2**	**5.5**	**7.4**	**5.5**
K (M^{-1})	$<1.10^3$	$5.10^5 \pm 2.10^5$	$9.10^5 \pm 3.10^5$	nd[a]	nd[b]	nd[b]	$1.5.10^5 \pm 0.5.10^5$	$8.69.10^9 \pm 1.10^2$	nd[b]
N	nd[b]	24.3 ± 0.4	11.6 ± 0.6	nd[b]	nd[b]	nd[b]	19.4 ± 0.6	11 ± 3	nd[b]

nd[a]: not determined due to a insufficient number of data points
nd[b]: not determined due to a low slope (very low affinity)

3.2 Effect of pH on protein-procyanidin interactions

Titration curves showing the enthalpy changes at pH 3.0, 5.5 and 7.5, due to interactions between α-lactalbumin and procyanidins of an average DP of 5.5, are shown in **Figure 2A** and for lysozyme in **Figure 2B**. As observed previously, the changes in enthalpy occurred in two stages. When α-lactalbumin was titrated with procyanidins, the exothermic effect at low ratios increased upon lowering the pH (**Figure 2A**). In contrast to the interactions with α-lactalbumin, the interactions between lysozyme and procyanidins become stronger at higher pHs (**Figure 2B** and **Table 1**). However, saturation of lysozyme, which is observed by a plateau in the trend, was reached at lower procyanidin concentrations at pH 5.5 than at pH 7.5 (**Figure 2B**). This indicates that more binding sites were available on lysozyme at low pH

than at high pH. At pH 5.5, lysozyme had a higher affinity for procyanidins than α-lactalbumin and also more binding sites seemed to be available on lysozyme than on α-lactalbumin (**Figure 2** and **Table 1**).

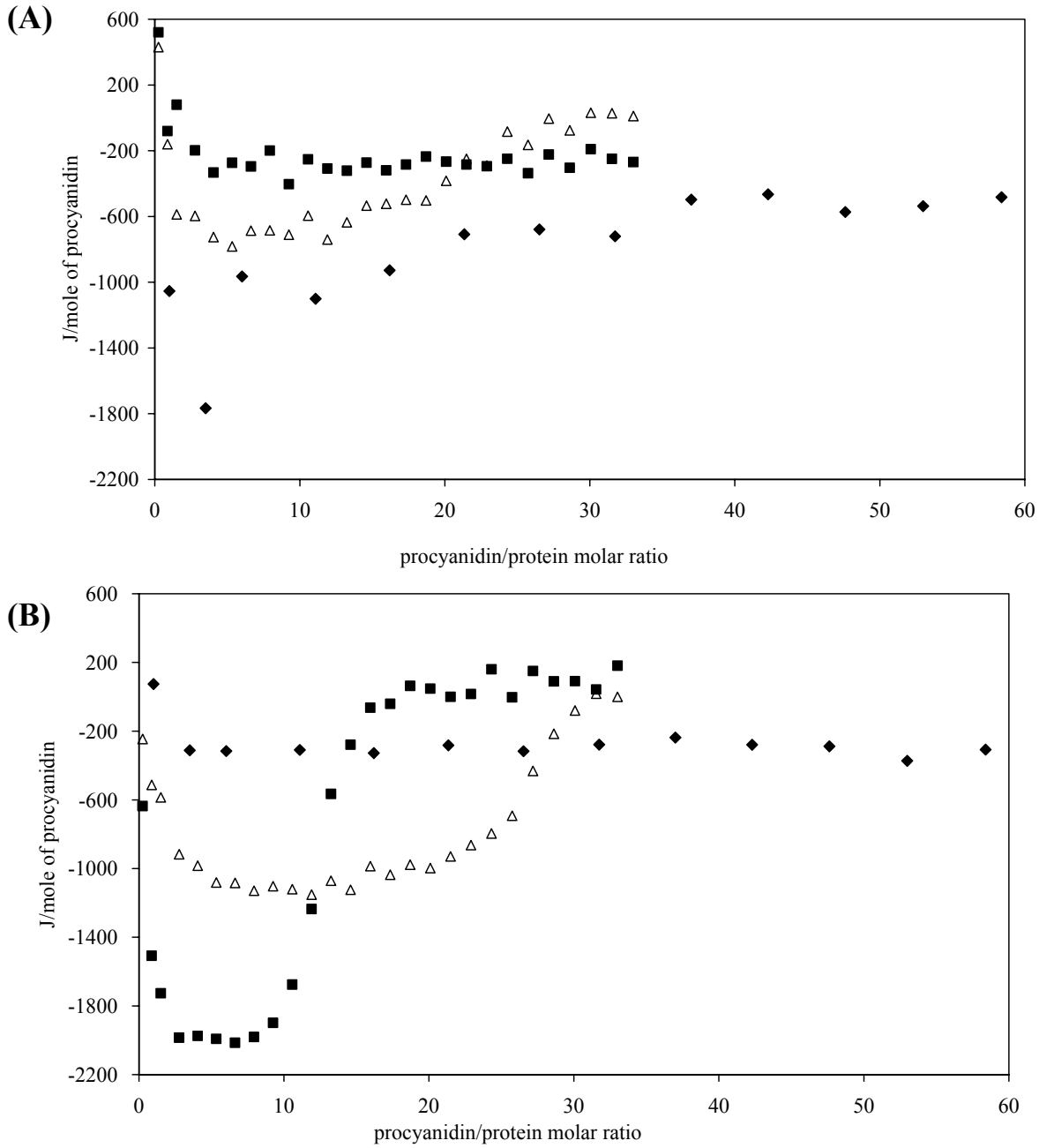


Figure 2. Isothermal titration calorimetry (25°C) of proteins titrated with procyanidins of DP5.5: (A) α-lactalbumin; (B) lysozyme. (◆) pH 3.0; (△) pH 5.5; (■) pH 7.5.

3.3 Effect of procyanidins on protein solubility

The effects of procyanidin interactions on protein solubility were also studied for BSA, because the low solubility of lysozyme at pH 7.0 in the presence of procyanidins prevented

the effects on foam properties to be studied. In the foam studies lysozyme was, therefore, replaced by BSA.

As presented in **Figure 3**, protein solubility decreased upon addition of procyanidins. The presence of procyanidins strongly decreased the solubility of α-lactalbumin to 0-10% between pH 3.0 and pH 6.0 (**Figure 3)**. At pH 4-5.5, the solubility of BSA decreased to 45-60% and was thus less affected and within a narrower pH-range than the solubility of α-lactalbumin (**Figure 3**). The solubility of lysozyme decreased to 40 % in the presence of procyanidins at pH 3, and was 0% at pH ≥ 6.0 (**Figure 3**).

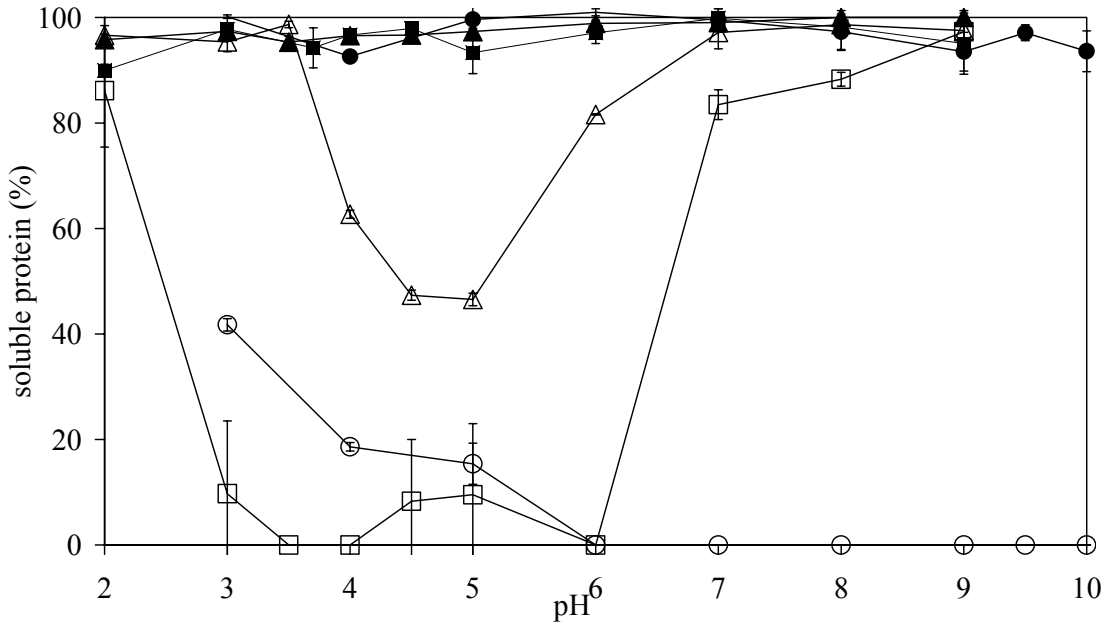


Figure 3. Protein (0.5% (w/v)) solubility as a function of pH, in the absence or presence of 5 moles of procyanidins (DP 7.4) per mole of protein: (■) α-lactalbumin, (●) lysozyme and (▲) BSA in the absence of procyanidins; (□) α-lactalbumin, (○) lysozyme and (△) BSA in the presence of procyanidins.

The effect of ionic strength on the protein solubility in α-lactalbumin-procyanidin (DP5.5) mixtures was measured after 2 and 72 hours of incubation at room temperature. After two hours at the three ionic strengths used, the solubility of α-lactalbumin had decreased from 95-100% in the absence of procyanidins to 18-21% in the presence of procyanidins (**Table 2**). No additional effects of prolonged incubation were observed (**Table 2**).

Table 2. Solubility of α-lactalbumin in the presence of procyanidin of an average DP of 5.5, as a function of temperature and ionic strength.

	percentage of protein solubility*			
	10°C	25°C		40°C
	2 hours	2 hours	72 hours	2 hours
27 mM	16 ± 1	20 ± 2	21 ± 5	27 ± 1
50 mM	nd#	18 ± 1	18 ± 4	nd#
100 mM	nd#	20 ± 1	18 ± 1	nd#

*percentage as a function of total protein content in samples
nd: not determined

When α-lactalbumin was incubated with procyanidins of DP5.5 for two hours at 10°C, 25 °C or 40°C, its solubility increased with temperature from 16%, at 10°C, to 27%, at 40°C (**Table 2**). This increase of solubility with temperature is low, but significant.

3.4 Effect of procyanidins on air-water interface and foam properties

The effects of procyanidins on air-water interface properties and foam properties of α-lactalbumin and BSA were studied at pH 4.0 and 7.0, with two moles of procyanidins (low ratio) and five moles of procyanidins (high ratio) per mole of protein. At both pHs the presence of procyanidins had neither an effect on the surface tension and elasticity at air-water interfaces of foams made with α-lactalbumin and BSA, nor on the average bubble diameter of foam made with BSA (data not shown). **Figure 4** shows the average bubble size as a function of time in foams made with α-lactalbumin under various conditions. It can be seen that the addition of procyanidins did not result in a decrease in the initial bubble diameter, but it was able to provide the bubbles in the foam with additional stability, presumably against Ostwald ripening, especially at the high ratio (**Figure 4B**). The curves in the presence of procyanidins in **Figure 4** indicate this stabilisation by showing a rather constant average bubble diameter, whereas in the absence of procyanidins and in the presence of low DP procyanidins a decrease in average bubble diameter is observed. The latter is due to coalescence of larger bubbles, indicating an overall coarsening of the foam. This difference in coarsening between foams made in the absence and presence of procyanidins is even clearer at pH 4.0, as presented in **Figures 5** and **6**.

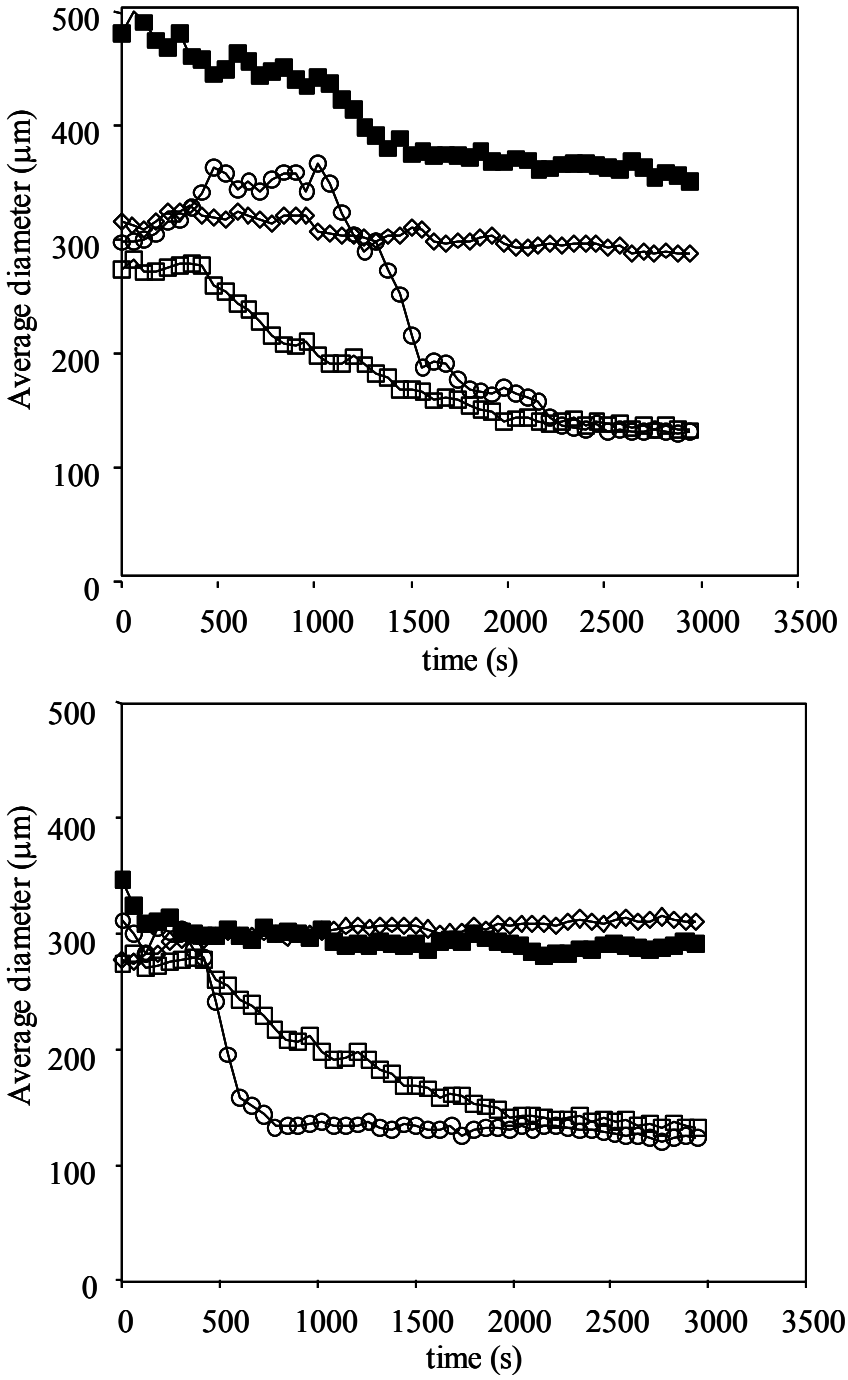


Figure 4. Average bubble diameter as a function of time for foam made from α-lactalbumin in the absence of procyanidins (□) or in the presence of procyanidins of an average DP of (○) 2.0, (◊) 5.0 and (■) 7.4, at pH 7.0. (A) procyanidin /protein molar ratio = 2; (B) procyanidin/protein molar ratio = 5.

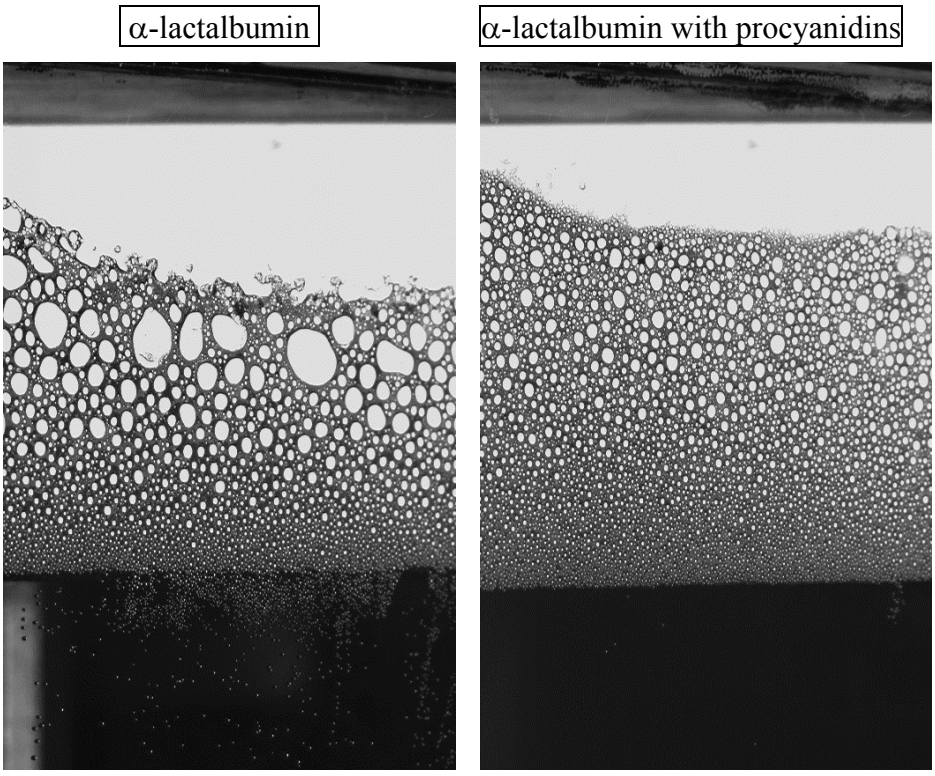


Figure 5. Images of foam from α-lactalbumin in the absence (left) and in the presence (right) of procyanidins of DP5 at pH 4.0 (after 49 min of storage).

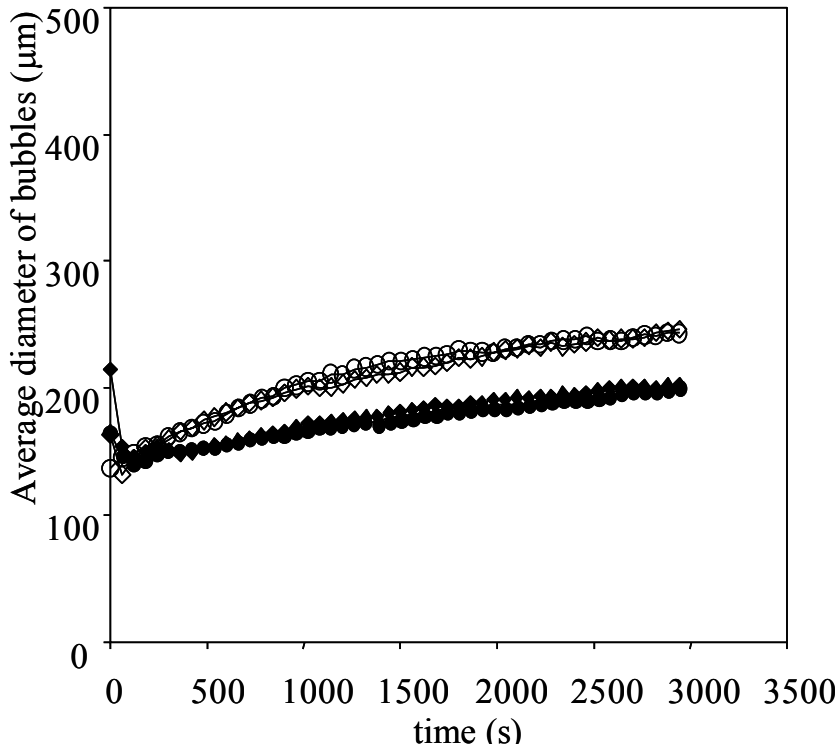


Figure 6. Average diameter of bubbles of foam from α-lactalbumin as a function of time in the absence and in the presence of procyanidins of an average DP of 5.0, at pH 4.0. (○,◇) in the absence and (●,◆) in the presence of procyanidins.

4. DISCUSSION

4.1 Characterisation of interactions between procyanidins and proteins

α-Lactalbumin does not show a pronounced affinity for epicatechin and procyanidin dimers, whereas the protein has a medium affinity for procyanidins of an average DP of 5.5 and a high affinity for procyanidins of an average DP of 7.4. The very low affinity of globular proteins for small procyanidins is in agreement with studies where BSA had an affinity < 1000 M^{-1} for epicatechin at pH 5.0, as measured by capillary electrophoresis (Papadopoulou and Frazier, 2004) and an affinity of 3000 M^{-1} for procyanidin dimers at pH 7.0, according to ultrafiltration measurements (Artz et al., 1987). The higher ability of large procyanidins to bind to proteins compared to small procyanidins may be explained by their multidentate character, which allows them to simultaneously bind several protein sites (Haslam, 1996).

Increasing the temperature has no effect on α-lactalbumin solubility in the absence of procyanidins, whereas it increased the amount of soluble α-lactalbumin in the presence of procyanidins. This could indicate that at higher temperatures α-lactalbumin interacts less strongly with procyanidins. Because hydrophobic interactions increase with higher temperature, whereas hydrophilic interactions decrease, this result indicates that hydrophilic interactions is the main driving force in α-lactalbumin-procyanidin interactions. Because no effect of ionic strength was observed, electrostatic interactions would not be the most important hydrophilic force controlling the interactions. Therefore, it can be proposed that hydrogen bonds are the main force in α-lactalbumin-procyanidin interactions. However, as only solubility is measured and not directly interactions, another possible explanation may be that the solubility of the aggregates increases with temperature.

Even after three days of incubation, increasing the ionic strength had no effect on the solubility of α-lactalbumin in the presence of procyanidins. Such a long incubation time was tested, because it has been observed in apple juice, where the main phenolic compound is the dimeric procyanidin B2, that a higher ionic strength decreases protein solubility after several days of incubation (Tajchakavit et al., 2001). The increase in turbidity could be due to interactions between phenolic compounds so forming insoluble complexes, but the exact reason is not clear (Tajchakavit et al., 2001).

As observed by ITC, at low molar procyanidin/protein ratios (≤ 5) the changes in enthalpy became more negative with increasing molar procyanidin/protein ratios. This

indicates that, at low ratios, once one procyanidin molecule is bound to the protein, the affinity of protein to bind another procyanidin molecule increases. The mechanism, therefore, shows positive cooperativity, as had also been observed for the interactions between gelatin and gallotannins and ellagitannins (Frazier et al., 2003). Positive cooperativity may be the result of a change in protein conformation upon ligand binding (Fersht, 1999). As shown in **Table 1**, the affinity of proteins for procyanidins increases when the pH is brought close to the isoelectric pH of the protein. This change in affinity as a function of pH is in agreement with other studies that have shown that the optimum pH for complex formation generally is 0.3-3 units below the pI of the protein (Naczk et al., 1996). In order to reach saturation of α-lactalbumin by procyanidins, the protein concentration used at pH 3.0 was four times lower than at higher pHs. Because more phenolics are required to precipitate protein in diluted protein solutions than in concentrated protein solutions (Haslam, 1996), the affinity observed at pH 3.0 is likely being under-estimated compared to the affinity measured at higher pHs. As observed by ITC, lysozyme interacted more strongly with procyanidins at pH 7.5 and pH 5.5 than α-lactalbumin, despite the fact that pH 5.5 is closer to the pI of α-lactalbumin than to that of lysozyme. This effect also became clearly apparent in the protein solubility measurements. α-Lactalbumin and lysozyme possess strongly similar sequences, but lysozyme has a lower hydrophobicity value than α-lactalbumin (Li-Chan, 1990). This seems to confirm the observation that the main forces driving the interactions between procyanidins and globular proteins are hydrogen bonding rather than hydrophobic interactions. Less interaction was expected, on a molar basis, with the low molecular weight α-lactalbumin than with the high molecular weight BSA, which possesses a pI similar to that of α-lactalbumin. BSA was indeed observed to have a higher affinity for proanthocyanidins than α-lactalbumin (Hagerman and Butler, 1981). Because procyanidins decrease the solubility of BSA to a lesser extent than the solubility of α-lactalbumin (**Figure 3**), these observations underline that the changes in solubility cannot entirely be ascribed to the strength of the interactions between proteins and procyanidins.

4.2 Effect of procyanidins on air-water interfaces and foam properties

At pH 7.0 and, in a more pronounced way at pH values close to the pI of α-lactalbumin, procyanidins of medium DP slowed down the coarsening of bubbles of foam made from α-lactalbumin, probably by stabilizing the foam against Ostwald ripening. They did not help to

create neither a higher foam volume, nor a initial higher number of bubbles of small diameter. Dimeric procyanidins seem to have no effect on foam properties because they do not easily simultaneously interact with several protein molecules, like larger procyanidins. However, catechin was observed to be able to improve foam ability and foam stability of foam made with proteins, possibly due to cross-linking of protein molecules by catechin molecules (Sarker et al., 1995). Such a mechanism was optimal at a molar catechin/protein ratio of 0.1. The fact that catechin affected foam properties (Sarker et al., 1995), whereas procyanidin dimers did not, can, therefore, be ascribed to the molar ratio that was used in the former study. But it can also be due to the nature of the protein-procyanidin couple studied or to the presence of Tween 20 used in the study of Sarker and co-workers (Sarker et al., 1995).

Summarizing, the interactions with procyanidins of medium DP are able to stabilise foam made with proteins, whereas those of smaller DP have no effect on protein foam properties, which can be related to the higher affinity of protein for procyanidins of medium DP than for smaller DP. These results show that procyanidins of medium DP can decrease the solubility of proteins, but may also have a positive role in foam stability.

LITERATURE CITED

Artz, W. E.; Bishop, P. D.; Dunker, A. K.; Schanus, E. G.; Swanson, B. G. Interaction of synthetic proanthocyanidin dimer and trimer with bovine serum albumin and purified bean globulin fraction G-1. *J. Agric. Food Chem.* **1987**, *35*, 417-421.

Asquith, T. N.; Butler, L. G. Interactions of condensed tannins with selected proteins. *Phytochemistry* **1986**, *25*, 1591-1986.

Baxter, N. J.; Lilley, T. H.; Haslam, E.; Williamson, M. P. Multiple interactions between polyphenols and a salivary proline-rich protein repeat result in complexation and precipitation. *Biochemistry* **1997**, *36*, 5566-5577.

Caessens, P. W. J. R.; Gruppen, H.; Visser, S.; Van Aken, G. A.; Voragen, A. G. J. Plasmin hydrolysis of β-casein: foaming and emulsifying properties of the fractionated hydrolysate. *J. Agric. Food Chem.* **1997**, *45*, 2935-2941.

Charlton, A. J.; Baxter, N. J.; Lokman, M. K.; Moir, A. J. G.; Haslam, E.; Davies, A. P.; Williamson, M. P. Polyphenol/peptide binding and precipitation. *J. Agric. Food Chem.* **2002**, *50*, 1593-1601.

De Freitas, V.; Mateus, N. Nephelometric study of salivary protein-tannin aggregates. *J. Sci. Food Agric.* **2001a**, *82*, 113-119.

De Freitas, V.; Mateus, N. Structural features of procyanidin interactions with salivary proteins. *J. Agric. Food Chem.* **2001b**, *49*, 940-945.

Escarpa, A.; González, M. C. High-performance liquid chromatography with diode-array detection for the determination of phenolic compounds in peel and pulp from different apple varieties. *J. Chromatogr. A* **1998**, *823*, 331-337.

Fersht, A. Conformational change, allosteric regulation, motors and work. In *Structure and mechanism in protein science: a guide to enzyme catalysis and protein folding*;; W.H. Freeman & Co.: New York, 1999; pp 289-323.

Frazier, R. A.; Papadopoulou, A.; Mueller-Harvey, I.; Kissoon, D.; Green, R. J. Probing protein-tannin interactions by isothermal titration microcalorimetry. *J. Agric. Food Chem.* **2003**, *51*, 5189-5195.

Guyot, S.; Marnet, N.; Drilleau, J.-F. Thiolysis-HPLC characterization of apple procyanidins covering a large range of polymerization states. *J. Agric. Food Chem.* **2001**, *49*, 14-20.

Hagerman, A. E.; Butler, L. G. Determination of protein in tannin-protein precipitates. *J. Agric. Food Chem.* **1980**, *28*, 944-947.

Hagerman, A. E.; Butler, L. G. The specificity of proanthocyanidin-protein interactions. *J. Biol. Chem.* **1981**, *256*, 4494-4497.

Hagerman, A. E.; Rice, M. E.; Ritchard, N. T. Mechanisms of protein precipitation for two tannins, pentagalloyl glucose and epicatechin$_{16}$ (4-->8) catechin (procyanidin). *J. Agric. Food Chem.* **1998a**, *46*, 2590-2595.

Hagerman, A. E.; Riedl, K. M.; Jones, G. A.; Sovik, K. N.; Ritchard, N. T.; Hartzfeld, P. W.; Riechel, T. L. High molecular weight plant polyphenolics (tannins) as biological antioxidants. *J. Agric. Food Chem.* **1998b**, *46*, 1887-1892.

Haslam, E. *Plant polyphenols: vegetable tannins revisited*; Phillipson, J.D.; Ayres, D.C.; Baxter, H., Eds.; Cambridge University Press: Cambridge, 1989, 167-192.

Haslam, E. Natural polyphenols (vegetable tannins) as drugs: possible modes of action. *J. Nat. Prod.* **1996**, *59*, 205-215.

Kumar, R.; Horigome, T. Fractionation, characterization, and protein-precipitating capacity of the condensed tannins from robinia pseudo acacia L. Leaves. *J. Agric. Food Chem.* **1986**, *34*, 487-489.

Le Bourvellec, C.; Guyot, S.; Renard, C. M. G. C. Non-covalent interaction between procyanidins and apple cell wall material. Part I. Effect of some environmental parameters. *Biochim. Biophys. Acta* **2004**, *1672*, 192-202.

Li-Chan, E. Hydrophobicity in food protein systems. In *Encyclopedia of food science and technology*; Y. H. Hui, Ed.; Wiley-Interscience: New York, 1990, 1429-1439.

Naczk, M.; Oickle, D.; Pink, D.; Shahidi, F. Protein precipitating capacity of crude canola tannins: effect of pH, tannin, and protein concentrations. *J. Agric. Food Chem.* **1996**, *44*, 2144-2148.

Oh, H. I.; Hoff, J. E.; Armstrong, G. S.; Haff, L. A. Hydrophobic interaction in tannin-protein complexes. *J. Agric. Food Chem.* **1980**, *28*, 394-398.

Papadopoulou, A.; Frazier, R. A. Characterization of protein-polyphenol interactions. *Trends Food Sci. Tech.* **2004**, *15*, 186-190.

Pierpoint, W. S. o-Quinones formed in plant extracts. Their reaction with bovine serum albumin. *Biochem. J.* **1969**, *112*, 619-629.

Riedl, K. M.; Hagerman, A. E. Tannin-protein complexes as radical scavengers and radical sinks. *J. Agric. Food Chem.* **2001**, *49*, 4917-4923.

Riou, V.; Vernhet, A.; Doco, T.; Moutounet, M. Aggregation of grape seed tannins in model wine - effect of wine polysaccharides. *Food Hydrocolloids* **2002**, *16*, 17-23.

Sanoner, P.; Guyot, S.; Marnet, N.; Molle, D.; Drilleau, J.-F. Polyphenol profiles of French cider apple varieties (*Malus domestica* sp.). *J. Agric. Food Chem.* **1999**, *47*, 4847-4853.

Santos-Buelga, C.; A., S. Review. Proanthocyanidins and tannin-like compounds - nature, occurrence, dietary intake and effects on nutrition and health. *J. Sci. Food. Agric.* **2000**, *80*, 1094-1117.

Sarker, D. K.; Wilde, P. J.; Clark, D. C. Control of surfactant-induced destabilization of foams through polyphenol-mediated protein-protein interactions. *J. Agric. Food Chem.* **1995**, *43*, 295-300.

Swain, T.; Hillis, W. E. The phenolic constituents of *Prunus domestica*. I. The quantitative analysis of phenolic constituents. *J. Sci. Food Agric.* **1959**, *10*, 63-68.

Tajchakavit, S.; Boye, J. I.; Bélanger, D.; Couture, R. Kinetics of haze formation and factors influencing the development of haze in clarified apple juice. *Food Res. Int.* **2001**, *34*, 431-440.

Torres, J. L.; Bobet, R. New flavanol derivatives from grape (Vitis vinifera) byproducts. Antioxidant aminoethylthio-flavan-3-ol conjugates from a polymeric waste fraction used as a source of flavanols. *J. Agric. Food Chem.* **2001**, *49*, 4627-4634.

Chapter 6

General discussion

Chapter 6

General discussion

1. NATURE OF THE INTERACTIONS

In this study, the interactions between globular proteins and phenolic compounds were studied and their effects on the functional properties of proteins were investigated in order to be able to modulate those protein properties important in food industry. The interactions were studied with monomeric phenolics and oligomeric phenolics. The interactions can be either non-covalent or covalent. As explained in **Chapter 1**, both types of interactions may theoretically become evident as a monodentate mechanism or a multidentate mechanism, which both may result in protein precipitation. Therefore, the occurrence and role of the monodentate and the multidentate mechanisms are discussed below.

1.1 Interactions with monomeric phenolics

Due to its high concentration in foods and its ubiquitous character, caffeoylquinic acid (CQA) was chosen as a representative of monomeric phenolics. It has been reported that CQA covalently reacts to a similar extent with whey proteins as caffeic acid, whereas ferulic acid reacts less (Rawel et al., 2000;Rohn et al., 2002). Flavonoids, such as quercetin, have been reported to have a slightly higher reactivity towards soy glycinin than CQA (Kroll et al., 2001). These results confirm that CQA can be seen as a representative of the monomeric phenolics in food.

1.1.1 Non-covalent interactions

To measure the affinities involved in non-covalent interactions with monomeric phenolics, isothermal titration calorimetry (ITC) and spectroscopic techniques were found not to be suitable methods because the interactions studied were too weak. For example, BSA contains one binding site for CQA with an affinity constant of 13-16 x 10^3 M^{-1} and other sites of such a low affinity that it was not possible to determine their affinity constants (**Chapter 2**). Equilibrium dialysis was also used (**Chapter 2**), but required many replicates because of the binding of phenolics to the membrane. In addition, it is time consuming. Gel filtration chromatography, using the technique of Hummel and Dreyer, and ultrafiltration were finally

the methods that were preferentially used to measure affinities of proteins for monomeric phenolics (**Chapter 2**).

Because of their small size, monomeric phenolic compounds cannot non-covalently interact via a multidentate mechanism. The non-covalent interactions observed between proteins and CQA only involve, therefore, a monodentate mechanism.

A monodentate mechanism has been proposed for the non-covalent interactions with monomeric phenolics, inducing protein precipitation (Haslam, 1989). However, as presented in **Chapter 2**, globular proteins have a low affinity for CQA and CQA does not decrease protein solubility. This implies that it is very unlikely that non-covalent interactions with monomeric phenolics will give rise to protein precipitation via a monodentate mechanism.

One of the richest sources of phenolic compounds, defatted sunflower meal, contains 2.5 % (w/w) CQA and 55 % (w/w) proteins (Gonzalez-Perez et al., 2002). The predominant protein in defatted sunflower meal is helianthinin (Gonzalez-Perez et al., 2002), and is present as a hexamer with a molecular weight of 360 kDa. This means that 1.3 molecules of CQA are present per protein. Considering the fact that BSA contains only one binding site for CQA with an affinity constant of 13-16 x 10^3 M^{-1} and other sites of very low affinity (**Chapter 2**), the molar ratios encountered in food are generally lower than the ratio required to non-covalently bind more than two molecules of CQA per molecule of protein and much lower than the ratio that would be required to precipitate protein. Therefore, despite the fact that the monodentate mechanism has been presented as a mechanism for protein precipitation (Haslam, 1989), it can be stated that monomeric phenolics will not affect the functional properties of proteins in food-like situations, when they interact non-covalently.

1.1.2 Covalent interactions

In contrast to the non-covalent interactions with CQA, covalent binding of CQA strongly decreases protein solubility and even induces a small degree of protein cross-linking (**Chapter 3**). To covalently interact with proteins, hydroxycinnamic acid derivatives such as CQA need to be oxidised into quinones by PPO, peroxidase (Oudgenoeg et al., 2001) or at alkaline pH. Because peroxidases require the presence of hydrogen peroxide (Matheis and Whitaker, 1984), their role in foods is limited compared to that of PPO. As explained in **Chapter 1**, the formation of quinones by PPO is restricted to o-diphenols and monophenols. However, phenolic compounds, which cannot be directly oxidised into quinones by polyphenol oxidase, can be oxidised by CQA quinones, depending on their redox potential

(Cheynier et al., 1988). This means that CQA oxidation induces oxydation of other phenolics and subsequent covalent interactions between these phenolics and proteins can occur.

The monodentate mechanism between proteins and CQA quinones occurs when CQA quinones do not react further with another protein molecule. Quinone dimers of CQA are able to covalently react with one lysine, tyrosine, histidine and tryptophan side-chain (**Chapter 4**), whereas monomers of CQA covalently react with one cysteine (Richard et al., 1991), histidine and tryptophan side-chains (**Chapter 4**). No reaction of CQA monomer was observed with lysine and tyrosine residues.

These results confirm the reaction mechanism between caffeic acid esters and an analogue of the lysine side-chain, butylamine, in which the phenolic compounds were observed to dimerise prior to reaction with amino compounds (Namiki et al., 2001). As explained in **Chapter 4**, the higher apparent reactivity of the oxidised CQA dimer compared to the oxidised CQA monomer could result from the lower redox potential of phenolic dimers compared to monomers (Singleton, 1987).

The multidentate mechanism, involving covalent quinone modification, has been observed as well. Proteins incubated with PPO are cross-linked in the presence or in the absence of exogenous phenolics, as presented in **Chapters 3 and 4**. This cross-linking can be partly explained by exogenous quinones and by direct cross-linking of tyrosine residues present in the protein chain. As presented in **Chapter 3**, a relatively low proportion of protein cross-linking is observed, whereas the decrease of protein solubility is high. This indicates that the decrease of solubility mostly comes from the monodentate mechanism, rather than the multidentate mechanism. This low extent of protein cross-linking can be explained by the fact that oxidised CQA mostly binds one single amino acid residue rather than two residues (**Chapter 4**).

Cross-linking also occurs in the absence of exogenous phenolics, due to the presence of tyrosine. Tyrosine, which is a mono-phenol, can be hydroxylated into an o-diphenol by PPO and subsequently oxidised into a quinone. In order to understand protein cross-linking in the absence of exogenous phenolic compounds, cross-linking between tyrosine side-chains was investigated. The products formed through the action of PPO on Boc-tyrosine had masses corresponding to dimers and trimers of Boc-tyrosine bearing one or two additional hydroxyl groups. Thus, PPO is able to hydroxylate Boc-tyrosine and to consequently induce protein cross-linking.

As explained in **Chapter 4**, PPO usually has an higher affinity for o-diphenols such as CQA than for monophenols such as tyrosine (García-Molina et al., 2005), PPO induces cross-links of proteins, in the presence of exogenous monomeric diphenolic compounds, by attachment of exogenous quinones to two amino acid residues rather than via cross-links of tyrosine. The presence of exogenous ferulic acid has been shown to limit the extent of protein dimerisation due to peroxidase, because the binding of ferulic acid limits further polymerisation of proteins once the ferulic acid is bound to the tyrosine residues of proteins (Oudgenoeg, 2004). A similar mechanism was not observed with CQA (**Chapter 3**). This can be due to the fact that once bound to an amino acid residue, in contrast to one molecule of ferulic acid, a dimer of CQA can still react with another amino acid residue.

1.2 Interactions with oligomeric phenolics

Proanthocyanidins were chosen as a model for oligomeric phenolic compounds as they represent the main subclass of condensed tannins present in food raw materials (**Chapter 5**). Proanthocyanidins are usually the tannins responsible for "tannin-like" behaviour observed in materials from plant origin (Haslam, 1989). As procyanidins constitute the most common member of proanthocyanidins (**Chapter 1**), they were chosen as a representative.

1.2.1 Non-covalent interactions

Due to their hydrophobicity, phenolic compounds, and especially oligomeric phenolic compounds, can bind to the column material or to membranes generally used for binding analysis experiments e.g. using ultrafiltration or chromatographic techniques. Therefore, the methods used in the study of monomeric phenolics interactions i.e. gel filtration chromatography, ultrafiltration and equilibrium dialysis were not suitable to study the interactions with oligomeric phenolics. As the interactions between proteins and oligomeric phenolics are relatively strong, the interactions between oligomeric phenolics and protein were studied by ITC (**Chapter 5**).

As shown in **Chapter 5**, the larger oligomeric procyanidins are able to interact with proteins over a relatively wide range of pH values. These interactions may involve both a monodentate and a multidentate mechanism, depending on the phenolic compound-protein ratio. The balance between these mechanisms will depend on the size of proanthocyanidins and on the proanthocyanidin-protein molar ratio. Both mechanisms can, however, not easily be distinguished. Globular proteins have much lower affinities for epicatechin and dimeric

procyanidins than for procyanidins of average DPs of 5.5 and 7.4 (**Chapter 5**). Precipitation of proteins by procyanidins of medium size was in some cases visible immediately after mixing the protein with the procyanidins (result not shown), indicating that non-covalent interactions with procyanidins take place instantaneously and strongly modify the physico-chemical properties of globular proteins.

1.2.2 Covalent interactions

Proanthocyanidins may covalently react following either a monodentate or a multidentate mechanism. Covalent binding of proanthocyanidins to proteins was not investigated, but, as with other phenolic compounds, may occur after oxidation (Guyot et al., 1996), or after depolymerization at low pH (Beart et al., 1985;Swain and Hillis, 1959).

A monodentate mechanism of covalent interactions with proanthocyanidins may also take place upon oxidation of proanthocyanidins as was studied for CQA. Due to their size, proanthocyanidins can, however, link to more than one protein molecule after oxidation, leading to a multidentate mechanism (Beart et al., 1985;Swain and Hillis, 1959). The covalent binding of proanthocyanidins due to oxidation can be expected to induce even more severe effects on protein functional properties than the covalent binding of monomeric phenolic compounds.

The covalent binding of proanthocyanidins to proteins due to their depolymerization at low pH has been reported to occur between procyanidins and the thiol group of cysteamine (Selga et al., 2004). Upon depolymerisation at low pH, the proanthocyanidin is split in two parts. The top part of the proanthocyanidin molecule bears a carbocation, which rapidly reacts with any nucleophilic group, thus also with those on proteins (**Figure 13, Chapter 1**), whereas the bottom part is released and is not reactive. Once bound to a protein, the proanthocyanidin may again depolymerise, thereby releasing a new top part bearing a carbocation, while the essentially unreactive bottom part remains bound to the protein. Therefore, proanthocyanidins degraded at low pH cannot subsequently react with several protein sites, and thus only react via a monodentate covalent mechanism. A mixture of covalent and non-covalent interactions may of course still occur. As the depolymerization of proanthocyanidins requires low pH values and high temperatures, the covalent binding to proteins due to depolymerization of proanthocyanidins at low pH may be restricted to certain food products.

2. DETECTION AND MODULATION OF PHENOLIC-PROTEIN INTERACTIONS IN FOODS

In order to modulate the properties of proteins in food through their interactions with phenolic compounds, the type of interactions involved should be taken into account**.**

2.1 Non-covalent interactions

Non-covalent interactions between proteins and phenolics in food products can be detected and avoided by using absorbents that bind to phenolics but not to proteins. For example, polyvinylpyrrolidone can be used to retain phenolics without interacting with proteins (Xu and Diosady, 2002).

Due to the high affinity between proteins and proanthocyanidins over a relatively wide pH range (**Chapter 5**), preventing non-covalent interactions between proanthocyanidins and globular proteins is not straightforward.

Because at pH values close to the isoelectric point of proteins, proteins show a higher affinity for proanthocyanidins than at pH values far from the pI, the interactions between proanthocyanidins and proteins may be modulated to a limited extent by pH. In addition, the decrease of α-lactalbumin solubility due to the presence of proanthocyanidins is somewhat more pronounced at low temperatures than at elevated temperatures (**Chapter 5**). This could be due to the fact that at elevated temperatures the interactions between proanthocyanidins and α-lactalbumin become weaker because they are dominated by hydrogen bonding (**Chapter 5**). However, in contrast to this, it has been reported that elevated temperatures increase the binding of proanthocyanidin dimers and trimers to BSA as measured by ultrafiltration (Artz et al., 1987). It thus seems that the solubility of protein-proanthocyanidin complexes may not always be a good measure of the strength and extent of the interactions involved. Because the extent and the forces involved in non-covalent interactions between proteins and oligomeric phenolics depend on the protein used and the nature of the oligomeric phenolics, these non-covalent interactions cannot easily be modulated in food. A way to avoid effects of oligomeric phenolics on protein properties is to decrease the amount of phenolic compounds by dephenolizing operations, e.g. using polyvinylpirrolidone, or organic solvents (Gonzalez-Perez et al., 2002;Jervis and Pierpoint, 1989;Xu and Diosady, 2002).

2.2 Covalent interactions

When monomeric diphenols such as CQA and other caffeic acid esters covalently react with the primary amino groups of proteins, a green pigment is formed (**Chapters 3 and 4;**Yabuta et al., 2001). Formation of a green colour formation can, therefore, indicate the occurrence of covalent interactions with hydroxycinnamic acids in food products.

In order to limit covalent interactions due to phenolic oxidation, the action of PPO should be neutralized, as explained in **Chapter 1**, and alkaline pHs should be avoided. Oxygen should be totally excluded as, even without agitation, CQA covalently reacts with proteins, at alkaline pH (**Figure 6, Chapter 2**).

2.3 Other factors

In complex food systems, compounds that are able to interact with proteins or with phenolic compounds also have to be taken into account. Carbohydrates, for example, have been observed to improve the solubility of oligomeric phenolic – protein complexes (De Freitas et al., 2003). Three reasons have been proposed for this positive effect of carbohydrates on protein solubility in the presence of phenolic compounds. Because carbohydrates are able to interact with phenolic compounds via hydrogen bonding and hydrophobic interactions (Riou et al., 2002), their interactions with phenolics may compete with protein-phenolic interactions (Mateus et al., 2004). They may also form a layer, at the periphery of phenolic-protein complexes and so improve the protein solubility (Mateus et al., 2004). Finally, certain carbohydrates, such as xanthan, are able to form complexes that may encapsulate phenolic compounds (Ozawa et al., 1987), thus preventing the oligomeric phenolic compounds from interacting with proteins.

3. APPLICATIONS AND LIMITATIONS OF PROTEIN-PHENOLIC INTERACTIONS IN FOODS

It can be stated that in food products the interactions responsible for the effects on functional properties of proteins are generally due to non-covalent interactions with oligomeric phenolics and to covalent interactions with both monomeric and oligomeric phenolics.

Interactions between phenolic compounds and globular proteins may induce protein cross-linking and precipitation. Such effects are usually undesirable in food products, but can be used to prepare certain new product formulations. For example, caffeic acid induces a dense

network in gelatin gels, which increases the mechanical strength and reduces the swelling of gels (Strauss and Gibson, 2004).

The hypothetic polymerization reaction of phenolic compounds and the covalent interactions between phenolic compounds and proteins seem responsible for enzymatic browning of e.g. wounded fruits (Rouet-Mayer et al., 1990). As CQA was not observed to form higher structures than CQA dimers (**Chapter 4**), and as dimers of caffeic acid ester are brown (Namiki et al., 2001), the enzymatic browning is hypothesised to mainly involve phenolic dimers, phenolic oligomers of low size and covalent phenolic compound-protein interactions, rather than hypothetical phenolic polymers of large size. Proteins that have been covalently modified by oxidised CQA are blue-green at neutral and alkaline pH (**Chapters 3 and 4**), which is usually not an appealing colour for most food products. They may, however, be used as natural colorants for new products. It is known that the covalent modification of proteins by CQA prevents or sometimes favors the cleavage of the proteins by digestive enzymes, depending on the enzyme involved (Kroll et al., 2003).

Additionally, proteins can be used to improve the taste of phenolic-containing food and to valorize the health-promoting potential of phenolics in food. One positive effect of protein-phenolic compound interactions is, that food proteins may decrease the astringency of phenolic compounds, because phenolics interacting with food proteins are less able to precipitate saliva proline-rich proteins. Therefore, if phenolic compounds bound to proteins still possess bio-functional properties, such as antioxidant properties, proteins could be helpful to obtain health-promoting food products that are not astringent. The question can be raised whether phenolic compounds are still strong antioxidants once bound to proteins. The covalent attachement of quercetin to BSA e.g. reduced its *in vitro* antioxidant activity (Rohn et al., 2004). The effects of non-covalent protein-phenolic interactions on the antioxidant properties of phenolics are not so clearly established, but a decrease of the antioxidant capacity of phenolics might also occur with non-covalent interactions (Arts et al., 2001). These results indicate that protein-phenolic interactions seem to decrease the *in vitro* antioxidant effects of free phenolics. However, these studies have been limited to *in vitro* tests and only to flavonoids and tannins. As monomeric phenolics weakly non-covalently interact with proteins (**Chapter 2**), their antioxidant value is not expected to be affected to a large extent by the presence of proteins. Furthermore, it would be interesting to determine whether, despite the decrease in antioxidant value of flavonoids and tannins measured *in vitro*, an increase in the uptake of antioxidative phenolics may be accomplished by the interactions

with proteins. It has even been determined that covalent conjugates of epicatechin with cysteamine presented a higher antioxidant value than epicatechin itself (Torres and Bobet, 2001). Since certain conjugates of phenolics and amino acids possess ionic groups, they have been proposed as tools to be used to modulate the action of antioxidative phenolics, e.g. to target the antioxidative molecule to selected cells (Selga et al., 2004;Torres and Bobet, 2001). Whereas phenolic compounds have restricted applications in modulating protein industrial properties, the results of the present study, and more particularly the observations on covalent interactions with amino acids, might, therefore, be useful to develop health-promoting conjugates from phenolic compounds and amino acids or peptides.

It can be concluded that, for food, non-covalent interactions are restricted to oligomeric phenolic compounds, despite the fact that they are usually more abundant than oligomeric phenolic compounds. For covalent interactions, the interactions with proteins are not restricted to lysine and cysteine residues, but also to tyrosine, histidine residues and to a lesser extent tryptophan residues interact. Phenolic compounds do not improve and sometimes worsen functional properties of proteins in food, except for certain properties where the creation of a protein-ligand network is desired.

LITERATURE CITED

Arts, M. J. T. J.; Haenen, G. R. M. M.; Voss, H.-P.; Bast, A. Masking of antioxidant capacity by the interaction of flavonoids with protein. *Food Chem. Toxicol.* **2001**, *39*, 787-791.

Artz, W. E.; Bishop, P. D.; Dunker, A. K.; Schanus, E. G.; Swanson, B. G. Interaction of synthetic proanthocyanidin dimer and trimer with bovine serum albumin and purified bean globulin fraction G-1. *J. Agric. Food Chem.* **1987**, *35*, 417-421.

Beart, J. E.; Lilley, T. H.; Haslam, E. Polyphenol interactions. Part 2. Covalent binding of procyanidins to proteins during acid-catalysed decomposition; observations on some polymeric proanthocyanidins. *J. Chem. Soc., Perkin Trans. 2* **1985**, 1439-1443.

Cheynier, V.; Osse, C.; Rigaud, J. Oxidation of grape juice phenolic compounds in model solutions. *J. Food Sci.* **1988**, *53*, 1729-1732.

De Freitas, V.; Carvalho, E.; Mateus, N. Study of carbohydrate influence on protein-tannin aggregation by nephelometry. *Food Chem.* **2003**, *81*, 503-509.

García-Molina, F.; Peñalver, M. J.; Fenoll, L. G.; Rodríguez-López, J. N.; Varón, R.; García-Cánovas, F.; Tudela, J. Kinetic study of monophenol and *o*-diphenol binding to *oxy*tyrosinase. *J. Mol. Catal.* **2005**, *32*, 185-192.

Gonzalez-Perez, S.; Merck, K. B.; Vereijken, J. M.; Van Koningsveld, G. A.; Gruppen, H.; Voragen, A. G. J. Isolation and characterization of undenatured chlorogenic acid free sunflower (*Helianthus annuus*) proteins. *J. Agric. Food Chem.* **2002**, *50*, 1713-1719.

Guyot, S.; Pellerin, P.; Brillouet, J.-M.; Cheynier, V. Inhibition of β-glucosidase (*Amygdalae dulces*) by (+)-catechin oxidation products and procyanidin dimers. *Biosci. Biotechnol. Biochem.* **1996**, *60*, 1131-1135.

Haslam, E. *Plant polyphenols: vegetable tannins revisited*; Phillipson, J.D.; Ayres, D.C.; Baxter, H., Eds.; Cambridge University Press: Cambridge, 1989, 230 p.

Jervis, L.; Pierpoint, W. S. Purification technologies for plant proteins. *J. Biotechnol.* **1989**, *11*, 161-198.

Kroll, J.; Rawel, H. M.; Rohn, S. Reactions of plant phenolics with food proteins and enzymes under special consideration of covalent bonds. *Food Sci. Technol. Res.* **2003**, *9*, 205-218.

Kroll, J.; Rawel, H. M.; Rohn, S.; Czajka, D. Interactions of glycinin with plant phenols - influence on chemical properties and proteolytic degradation of the proteins. *Nahrung/Food* **2001**, *45*, 388-389.

Mateus, N.; Carvalho, E.; Luis, C.; de Freitas, V. Influence of the tannin structure on the disruption effect of carbohydrates on protein-tannin aggregates. *Anal. Chim. Acta* **2004**, *513*, 135-140.

Namiki, M.; Yabuta, G.; Koizumi, Y.; Yano, M. Development of free radical products during the greening reaction of caffeic acid esters (or chlorogenic acid) and a primary amino compound. *Biosci. Biotechnol. Biochem.* **2001**, *65*, 2131-2136.

Oudgenoeg, G. Peroxidase catalysed conjugation of peptides, proteins and polysaccharides via endogenous and exogenous phenols. Ph.D. thesis, Wageningen, 2004.

Oudgenoeg, G.; Hilhorst, R.; Piersma, S. R.; Boeriu, C. G.; Gruppen, H.; Hessing, M.; Voragen, A. G. J.; Laane, C. Peroxidase-mediated cross-linking of a tyrosine-containing peptide with ferulic acid. *J. Agric. Food Chem.* **2001**, *49*, 2503-2510.

Ozawa, T.; Lilley, T. H.; Haslam, E. Polyphenol interactions: astringency and the loss of astringency in ripening fruit. *Phytochemistry* **1987**, *26*, 2937-2942.

Rawel, H. M.; Rohn, S.; Kroll, J. Reactions of selected secondary plant metabolites (glucosinolates and phenols) with food proteins and enzymes -Influence on physico-chemical protein properties, enzyme activity and proteolytic degradation. *Recent Res. Devel. Phytochem.* **2000**, *4*, 115-142.

Richard, F. C.; Goupy, P. M.; Nicolas, J. J.; Lacombe, J.-M.; Pavia, A. A. Cysteine as an inhibitor of enzymatic browning. 1. Isolation and characterization of addition compounds formed during oxidation of phenolics by apple polyphenol oxidase. *J. Agric. Food Chem.* **1991**, *39*, 841-847.

Riou, V.; Vernhet, A.; Doco, T.; Moutounet, M. Aggregation of grape seed tannins in model wine - effect of wine polysaccharides. *Food Hydrocolloids* **2002**, *16*, 17-23.

Rohn, S.; Rawel, H. M.; Kroll, J. inhibitory effects of plant phenols on the activity of selected enzymes. *J. Agric. Food Chem.* **2002**, *50*, 3566-3571.

Rohn, S.; Rawel, H. M.; Kroll, J. Antioxidant activity of protein-bound quercetin. *J. Agric. Food Chem.* **2004**, *52*, 4725-4729.

Rouet-Mayer, M.-A.; Ralambosoa, J.; Philippon, J. Roles of *o*-quinones and their polymers in the enzymic browning of apples. *Phytochemistry* **1990**, *29*, 435-440.

Selga, A.; Sort, X.; Bobet, R.; Torres, J. L. Efficient one pot extraction and depolymerization of grape (*Vitis vinifera*) pomace procyanidins for the preparation of antioxidant thio-conjugates. *J. Agric. Food Chem.* **2004**, *52*, 467-473.

Matheis, G.; Whitaker, J. R. Modification of proteins by polyphenol oxidase and peroxidase and their products. *J. Food Biochem.* **1984**, *8*, 137-162.

Singleton, V. L. Oxygen with phenols and related reactions in musts, wines and model systems: observations and practical implications. *Am. J. Enol. Vitic.* **1987**, *38*, 69-77.

Strauss, G.; Gibson, S. M. Plant phenolics as cross-linkers of gelatin gels and gelatin-based coacervates for use as food ingredients. *Food Hydrocolloids* **2004**, *18*, 81-89.

Swain, T.; Hillis, W. E. The phenolic constituents of *Prunus domestica*. I. The quantitative analysis of phenolic constituents. *J. Sci. Food Agric.* **1959**, *10*, 63-68.

Torres, J. L.; Bobet, R. New flavanol derivatives from grape (*Vitis vinifera*) byproducts. Antioxidant aminoethylthio-flavan-3-ol conjugates from a polymeric waste fraction used as a source of flavanols. *J. Agric. Food Chem.* **2001**, *49*, 4627-4634.

Xu, L.; Diosady, L. L. Removal of phenolic compounds in the production of high-quality canola protein isolates. *Food Res. Int.* **2002**, *35*, 23-30.

Yabuta, G.; Koizumi, Y.; Namiki, K.; Hida, M.; Namiki, M. Structure of green pigment formed by the reaction of caffeic acid esters (or chlorogenic acid) with a primary amino compound. *Biosci. Biotechnol. Biochem.* **2001**, *65*, 2121-2130.

Summary

Summary

Phenolic compounds are compounds present in foods and raw materials from plant sources. Because polyphenols are able to interact with proteins, they can modify the functional properties of proteins, such as solubility. In order to be able to control the functional properties of proteins important in food industry i.e. the globular proteins, this study was aimed at characterizing the interactions between globular proteins and phenolic compounds. Both covalent and non-covalent interactions between monomeric and multimeric phenolic compounds and globular proteins were investigated. Their effects on the physico-chemical and functional properties of proteins were studied.

Chapter 1 introduces the subject with a literature overview concerning the occurrence and the classification of polyphenols. The classification is based on the number of carbon atoms. It also presents knowledge on the interactions of phenolics with each other, and hypothesis and features on protein-phenolic interactions described in literature. Caffeoylquinic acid (CQA, chlorogenic acid) and procyanidins are described as they were chosen as representatives of the monomeric phenolic compounds and oligomeric phenolic compounds, respectively. α-Lactalbumin, lysozyme and BSA (bovine serum albumin) were chosen as models for globular proteins.

Chapter 2 describes the non-covalent interactions between CQA and the proteins studied and their effects on protein properties. Proteins, when they non-covalently interact, have a low affinity for CQA. These non-covalent interactions between proteins and CQA are shown to involve a negative cooperativity of binding. The pH and ionic strength do not affect the affinity between CQA and proteins. In contrast, the temperature strongly influenced the amount of CQA bound to bovine serum albumin: the binding decreased with increasing temperature. The importance of hydrogen bonding as a main force driving these interactions is discussed. The non-covalent CQA-protein interactions were observed to have no effect on protein solubility. At high molar CQA/protein ratios, CQA increased the denaturation enthalpy and denaturation temperature of BSA. However, heating was shown to create covalent CQA-protein interactions.

In **Chapter 3**, the covalent interactions between CQA oxidised by polyphenol oxidase (PPO), at pH 6.0, and proteins are characterized and compared with non-enzymatically induced covalent modifications at alkaline pH (pH 9.0). Both ways of modification were shown to

result in protein modification mainly via dimeric rather than via monomeric CQA quinones. These modifications led to a decrease in both the number of free primary amino groups and the isoelectric point of the proteins. The covalent modifications of proteins with CQA strongly reduced the protein solubility at pH ≤ pI and induced some protein dimerisation. Modification drastically reduced the solubility of lysozyme and to a lower extent those of α-lactalbumin and BSA. These results indicate that the same kind of modifications occur at pH 6.0 as at pH 9.0. No effect of the interactions on foam properties was observed.

The observations presented in **Chapter 3** concerning covalently modified proteins are clarified in **Chapter 4** by studying amino-acid side-chain reactivity. Mass spectrometry analysis of mixtures of PPO-oxidised CQA and amino acid protected at their N-terminal group indicated that covalent reactions occur between a dimer of CQA quinone and lysine, tyrosine, histidine and tryptophan side-chains. Monomers of oxidised CQA were observed to react with histidine and tryptophan side-chains. Lysine and tyrosine side-chains were observed to be more reactive than the histidine side-chain. The tryptophan side-chain exhibited a lower reactivity than the histidine side-chain. No reaction products were observed between CQA and the side-chain of serine and threonine. Molecular masses corresponding to cross-linking of CQA with two lysine residues were found indicating that crosslinking of protein is possible. Hypothetical structures are proposed in **Chapter 4**. The results of **Chapter 4** confirm the observations on protein studies presented in **Chapter 3** that reactions of CQA encompass not only lysine and cysteine residues and that protein crosslinking by CQA is possible.

Chapter 5 describes the non-covalent interactions between procyanidins of various degrees of polymerisation (DP) and globular proteins, concerning the affinities between procyanidins and proteins. The effects of these interactions on protein solubility and foam properties were also investigated. Proteins showed a medium affinity for procyanidins of an average DP of 5.5, but weakly interacted with epicatechin and procyanidin dimer. A positive cooperativity of binding was observed with procyanidins of DP 5.5 and 7.4 at low molar procyanidin/protein ratios. As expected, the affinity of α-lactalbumin and lysozyme for procyanidins increased when the pH was close to the isoelectric point. Procyanidins of DP 5.5 were shown to strongly decrease the solubility of lysozyme and to a smaller extent the solubility of α-lactalbumin and BSA. The importance of hydrogen bonding as a main force driving the

interactions between α-lactalbumin and procyanidins is hypothesized. A slight positive effect of procyanidins of DP 5.5 and 7.4 on foam stability was observed.

In **Chapter 6**, the mechanisms of protein-polyphenol interactions are discussed. The effects of the non-covalent and covalent interactions on protein functional properties are compared. The importance of oligomeric phenolics compared to monomeric phenolics on protein functional properties is underlined. The different parameters that can be used to modulate the interactions between proteins and polyphenols are discussed. In addition, the role of carbohydrates in improving the solubility of phenolic compound-protein complexes is explained. Finally, the results of this thesis are discussed for their application in food.

Samenvatting

Fenolische verbindingen komen in veel voedingsmiddelen en in plantenmateriaal voor. De interactie van de fenolische verbindingen met eiwitten, kan de functionele eigenschappen van eiwitten beïnvloeden. Om de functionele eigenschappen van voor de levensmiddelenindustrie van belang zijnde eiwitten (de globulaire eiwitten) te kunnen beheersen, was het doel van dit onderzoek om de interactie tussen globulaire eiwitten en fenolische verbindingen te karakteriseren. Zowel de covalente als niet-covalente interactie tussen de monomere- en multimere-fenolische verbindingen met globulaire eiwitten is onderzocht. Daarnaast is het effect van de interactie op de fysisch-chemisch en functionele eigenschappen van de eiwitten bestudeerd.

In het inleidende hoofdstuk (hoofdstuk 1) wordt een literatuur overzicht gegeven van het voorkomen en de classificatie van fenolische verbindingen. Deze classificatie is gebaseerd op het aantal koolstofatomen van het polyfenol. In dit hoofdstuk wordt aandacht besteed aan de onderlinge interacties van fenolische verbindingen en de hypotheses en kenmerken van polyfenol-eiwit interacties zoals ze beschreven staan in de literatuur. Chlorogeenzuur (CQA, caffeoylquinic acid) werd geselecteerd als model voor monomere fenolische verbindingen en procyanidines werden gekozen als model voor oligomere fenolische verbindingen. α-Lactalbumine, lyzozym en BSA (bovine serum albumine) werden gekozen als model voor de globulaire eiwitten.

Hoofdstuk 2 beschrijft de niet-covalente interactie tussen CQA en eiwit en de effecten van de interactie op de eigenschappen. Eiwitten hebben een lage affiniteit voor CQA voor de vorming van niet-covalente bindingen. Naarmate er meer CQA door niet-covalente interactie aan het eiwit gebonden worden nam de affiniteit voor CQA af. De pH en ionsterkte hadden geen effect op de interactie tussen CQA en eiwit. De temperatuur beïnvloedde de hoeveelheid CQA dat aan BSA bond: de binding werd minder naarmate de temperatuur hoger werd. Het belang van waterstofbrugvorming als een drijvende kracht achter deze interactie wordt bediscussieerd. De niet-covalente CQA-eiwit interacties lieten geen effect op de oplosbaarheid van de eiwitten zien. Bij hogere CQA/eiwit ratio's werd de denaturatie enthalpie en de denaturatie temperatuur van BSA hoger. Verhitten resulteerde in covalente CQA/eiwit interacties.

In hoofdstuk 3 wordt de covalente interactie tussen CQA en eiwit na oxidatie door polyphenol oxidase (PPO) bij pH 6.0 gekarakteriseerd en vergeleken met de interactie geïnduceerd door niet-enzymatische oxidatie bij pH 9.0. In beide gevallen bleken de modificatie reacties vooral te verlopen via de dimere chinonen en niet via het monomere CQA chinon. Deze modificaties leidden zowel tot een afname in het aantal vrije primaire aminogroepen als tot een verlaging van het isoelectische punt. De covalente modificatie met CQA verlaagde de oplosbaarheid van de eiwitten bij een pH < pI sterk en induceerde enige dimerisatie van de eiwitten. Modificaties leidden tot een sterke verlaging van de oplosbaarheid van lysozym en in mindere mate tot een verlaging van de oplosbaarheid van α-lactalbumine en BSA. Deze resultaten suggereren dat zowel bij pH 6.0 als bij pH 9.0 dezelfde modificatie reacties optreden. Er werden geen effecten van de interacties op de schuimstabiliteit waargenomen.

De resultaten van de covalent gemodificeerde eiwitten die in hoofdstuk 3 zijn beschreven worden in hoofdstuk 4 nader belicht door de reactiviteit van de zijketens van aminozuren te bestuderen. Analyse van de reactieproducten van het met PPO-geoxideerde CQA met N-terminaal geblokkeerde aminozuren met behulp van massa spectroscopie laat zien dat covalente reacties plaats vinden tussen het dimeer van het CQA chinon en de lysine, tyrosine, histidine en tryptofaan zijketens. Monomeren van geoxideerd CQA blijken alleen met de histidine en tryptofaan zijketens te reageren. De reactiviteit van de lysine en tyrosine zijketens blijkt hoger dan de reactiviteit van de histidine zijketen. De reactiviteit van de tryptofaan zijketen is lager dan de reactiviteit van de histidine zijketen. Samenvoegen van CQA en N-terminaal geblokkeerd serine of threonine resulteerde niet in reactie producten. Het observeren van molekuulgewichten die overeenkomen met CQA gecrosslinked met twee lysine residuen impliceerden dat CQA kan functioneren als crosslinker tussen eiwitten. Een hypothetische structuur is beschreven in hoofdstuk 4. De resultaten beschreven in hoofdstuk 4 bevestigen de observaties uit hoofdstuk 3: de reacties met CQA omvatten niet alleen lysine en cysteine residuen en crosslinken van eiwit met behulp van CQA is mogelijk.

Hoofdstuk 5 beschrijft de niet-covalente interacties tussen procyanidines met een verschillende polymerisatie graad (DP) en globulaire eiwitten met betrekking tot de affiniteit tussen procyanidines en eiwitten. De effecten van deze interactie op eiwit stabiliteit en schuimeigenschappen zijn ook bestudeerd. Eiwitten lieten een gemiddelde affiniteit voor procyanidines met een gemiddelde DP van 5.5 zien. Echter, de interactie met epicatechine en met dimeren van procyanidines was zwak. Bij een lage molaire procyanidines/eiwit ratio werd een positief effect van procyanidines op de binding tussen procyanidines en eiwitten

waargenomen. Zoals verwacht, nam de affiniteit van α-lactalbumine en lysozyme voor procyanidines toe naarmate de pH dichter bij het isoelectrisch punt van het eiwit kwam te liggen. Procyanidines met een DP 5.5 verlaagden de oplosbaarheid van lysozym sterk en de oplosbaarheid van α-lactalbumine met BSA was ook lager. Waterstofbruggen worden verondersteld de belangrijkste drijvende kracht achter de interactie tussen α-lactalbumine en procyanidines te zijn. Procyanidines met een DP van 5.5 en 7.4 vertoonden een klein positief effect op de schuimstabiliteit.

In hoofdstuk 6 worden de mechanismen bediscussieerd die ten grondslag liggen aan de eiwit-polyfenol interacties. De effecten van de niet-covalente en covalente interacties op de functionele eigenschappen van eiwitten worden vergeleken. Hierbij ligt de nadruk op het belang van de oligomere fenolen met betrekking tot de functionele eigenschappen van eiwitten in vergelijking met de monomere fenolen. De verschillende parameters die de interactie tussen eiwitten en polyfenolen kunnen beïnvloeden komen aan de orde. Daarnaast wordt toegelicht welke invloed koolhydraten kunnen hebben bij de verdere verbetering van de oplosbaarheid van fenol-eiwit complexen. Tot slot wordt aangegeven hoe de resultaten die beschreven staan in dit proefschrift door de levensmiddelenindustrie kunnen worden toegepast.

Résumé

Les composés phénoliques sont présents dans les aliments et les matières premières d'origine végétale. Les polyphénols étant capables d'interagir avec les protéines, ils peuvent modifier leurs propriétés fonctionnelles telle que la solubilité. Dans le but de contrôler les propriétés fonctionnelles des protéines importantes dans l'industrie agro-alimentaire, en particulier les protéines globulaires, cette étude visait à caractériser les interactions entre protéines globulaires et composés phénoliques. Plus précisément, les interactions covalentes et non covalentes entre les composés phénoliques monomériques et multimériques et les protéines globulaires furent étudiées.

Le **chapitre 1** présente le sujet avec une vue d'ensemble de la littérature concernant leur présence et la classification des polyphénols. Les connaissances sur les interactions des composés phénoliques entre eux et sur les interactions protéines-polyphénols sont décrites. L'acide caféoylquinique (CQA, acide chlorogénique) et les procyanidols furent respectivement choisis comme représentants des composés phénoliques monomériques et oligomériques. L'α–lactalbumine, le lysozyme et la BSA (sérum albumine bovine) furent choisis comme modèles de protéines globulaires.

Le **chapitre 2** décrit les interactions non covalentes entre le CQA et les protéines étudiées, ainsi que leurs effets sur les propriétés des protéines. Les protéines, quand elles interagissent de manière non covalente, ont une faible affinité pour le CQA. Ces interactions non covalentes font intervenir une coopérativité négative. Le pH et la force ionique n'affectent pas l'affinité entre le CQA et les protéines. Au contraire, la température influence fortement la quantité de CQA liée à la BSA : l'attachement diminue avec l'augmentation de la température. L'importance des liaisons hydrogènes comme force principale contrôlant ces interactions est discutée. Il a été observé que les interactions non covalentes entre le CQA et les protéines n'ont aucun effet sur la solubilité des protéines. A des ratios CQA/protéines élevés, le CQA augmente l'enthalpie de dénaturation et la température de dénaturation de la BSA. Cependant, il fut observé que le chauffage créé des interactions covalentes entre le CQA et les protéines.

Dans le **chapitre 3**, les interactions covalentes entre le CQA oxydé par la polyphénol oxydase (PPO), à pH 6.0, et les protéines sont caractérisées et comparées avec les interactions covalentes induites de manière non enzymatique à pH alcalin (pH 9.0). Il fut démontré que les

deux voies de modification résultaient en une modification des protéines via des quinones de CQA dimériques plutôt que monomériques. Ces modifications conduisirent à la fois à une diminution du nombre d'acides aminés primaires et à une diminution du point isoélectrique (pI) des protéines. Les modifications covalentes des protéines avec le CQA diminuèrent fortement la solubilité des protéines à des pH inférieurs ou égaux au pI et induisirent une dimérisation partielle des protéines. La modification covalente des protéines avec le CQA diminue drastiquement la solubilité du lysozyme et à un moindre degré celle de l'α–lactalbumine et de la BSA. Ces résultats indiquent que le même genre de modifications intervient à pH 6.0 qu'à pH 9.0. Aucun effet de ces interactions ne fut observé sur les propriétés moussantes.

Les observations sur les protéines modifiées de manière covalentes du **chapitre 3** sont clarifiées dans le **chapitre 4**, où la réactivité des chaînes latérales des acides aminés fut étudiée. L'analyse par spectrométrie de masse des mélanges de CQA oxydé par la PPO et d'acides aminés ayant leur groupe N-terminal protégé indique que des réactions covalentes interviennent entre un dimère d'une quinone de CQA et la chaîne latérale de la lysine, la tyrosine, l'histidine et le tryptophane. Il a été observé que des monomères de CQA oxydé sont capables d'interagir avec la chaîne latérale de l'histidine et du tryptophane. Les chaînes latérales de lysine et de tryptophane furent plus réactives que celle de l'histidine. Aucun produit de réaction ne fut observé entre le CQA et la chaîne latérale de la sérine et de la thréonine. Les masses moléculaires obtenues correspondraient à un cross-linking d'une molécule de CQA avec deux résidus de lysine, pour lequel d'hypothétiques structures sont proposées. Les résultats du **chapitre 4** confirment les observations présentées au **chapitre 3**, indiquant que les réactions avec le CQA ne font pas intervenir uniquement les résidus lysine et cystéine, et que le cross-linking des protéines est possible.

Le **chapitre 5** décrit les interactions non covalentes entre les procyanidols de différents degrés de polymérisation (DP) et les protéines globulaires. En outre, les effets de ces interactions sur la solubilité et sur les propriétés moussantes des protéines ont aussi été étudiés. Les protéines ont montré une affinité moyenne pour les procyanidols de DP moyen de 5.5, tandis qu'elles ont faiblement interagit avec l'épicatéchine et le dimère de procyanidol. Une coopérativité positive de liaison fut observée entre les procyanidols de DP 5.5 et 7.4 à des ratios procyanidol/protéines bas. Comme attendu, l'affinité pour l'α–lactalbumine et le lysozyme augmenta quand le pH était proche du pI. Il a été montré que les procyanidols de DP 5.5 diminuent fortement la solubilité du lysozyme et, à un moindre degré, la solubilité de

l'α–lactalbumine et de la BSA. L'importance des liaisons hydrogènes comme force principale contrôlant les interactions entre l'α–lactalbumine et les procyanidols est proposée. Un faible effet positif des procyanidols de DP 5.5 et 7.4 sur la stabilité de la mousse a été observé.

Dans le **chapitre 6**, les mécanismes des interactions entre protéines et polyphénols sont discutés. Les effets des interactions non covalentes et covalentes sur les propriétés fonctionnelles des protéines sont comparés et l'importance des composés phénoliques oligomériques par rapport à celle des composés phénoliques monomériques sur les propriétés fonctionnelles des protéines est soulignée. De plus, les différents paramètres qui peuvent être utilisés pour moduler les interactions entre protéines et polyphénols sont discutés. Le rôle des sucres dans l'amélioration de la solubilité des complexes protéine–composé phénolique est également expliqué. Enfin, les résultats de cette thèse sont discutés pour leurs applications dans les aliments.

Acknowledgements

Finally! When I decided to do this project, I did not realise that a Ph.D. project could be sometimes a mix of a law of Murphy (« everything that can go wrong will go wrong ») and of the principle of video games (« same player, play again »). But the help and the kindness of the colleagues of the LMC lab helped me to keep the « faith » to reach the goal. So, I want to warmly thank all of you for your advices, support, smile and good mood.

I would like to thank my promotors, Fons Voragen, Harry Gruppen and Ton Visser for offering me this Ph.D. position and for believing in me despite the difficulties of the project encountered at the beginning. Harry and Gerrit van Koningsveld, thank you for the discussions and comments to improve the chapters. Gerrit, your very good knowledge of many experimental techniques was helpful. Sander Piersma, I am grateful for your advices and optimism. Thomas Haertlé, merci à vous pour l'intérêt que vous accordez à vos anciens étudiants.

I also want to thank persons from other labs who helped me during this project : Wim Lichtendonk, Bertus Dunnewind and Martin Bos (TNO, Zeist) for the discussions and practical help with the air-water interfaces and foam experiments, all the colleagues of the Station Cidricole (INRA, Le Rheu) pour l'accueil, l'aide expérimentale et les discussions, plus particulièrement Catherine Renard, Nathalie Marnet, Sylvain Guyot, Stéphane Bernillon et Alain Barron, and also thank to Prof. de Groot for the useful discussion on chemical reactions and to Anton Korteweg for his kind help with ITC.

As previously written, I am very grateful to each of you from the LMC department. Karin, thank you to have accepted to be my paranymph and bedankt voor de vertaling ! I also would like to thank more particularly for their help: Jolanda van den B., Hanem, Margaret, Jolan, Harmen, Hans, Jan, René K., René V., Peter W., Ben, Edwin and Bas. I want also to thanks the students, Gertjan Blom and Feng Li: thanks to have work (very hard !) on this project. A big thank to the room-mates for the good mood : Mingwey, Mirjam, Evelien, Lidvin, Annemarie, Koen, Jean-Paul, Johan and Jacques. Thanks also for the support and the nice time spent : Sergio (the philosophy in the humor), Wil, Julia, Hauke, Gerd-Jan, Renate, without forgetting Aagje (thanks for the rawing lessons !), and many more…

Steph G., « tu t'rappelles dans l'temps quand on faisait, comment ça s'appelait d'jà, ah oui, une thèse… », je ne risque pas d'oublier tous ces moments ! Un grand merci à toi pour tout. Lynn and Kerensa, I won't forget the great trip to Asia. Family Heng, I wait for your visit…Lynn, good that we'll be neighbours! Nathalie, merci pour l'aide, j'espère que tu ne vas pas partir dans un pays encore plus éloigné ! Laurice, alors finalement tu as découvert dans les indigènes locaux un intérêt que tu ne soupçonnais pas ? (;o)

To all my friends, I thank you for your support and fun. Fred et Jeff, vous êtes des « frères » pour moi, merci pour votre joie de vivre et votre soutien. Franck et Véro (« Maison de la Pizza, bonjour »), un grand merci d'offrir des soirées ambiance familiale ou ambiance années 80, elles m'ont bien aidé à apprécier la vie Wageningenienne. Catriona, heel bedankt to help us to discover the dutch gezelligheid ! Will and Yaité, gracias/merci for your kindness and nice dinners, it was a pleasure to have you as neighbours. A big thank also to : Miki (and his plant), Totof (and his dance of the ducks), Flo from Delft (and her laugh), Sam, David, Nico, Manu, Déa, Jérôme and Karen (and their visit to Gouda-Land).

Enfin, je profite de cette opportunité pour exprimer ma gratitude envers ma grand-mère (je vais pouvoir te voir plus souvent maintenant), la famille Prigent-Caré, et plus particulièrement envers mes parents et ma soeur, pour leur gentillesse depuis toujours. Je sais que j'ai beaucoup de chance d'avoir des parents et une soeur tels que vous. Joakin et Kilian, vous ne savez pas encore lire, mais je devrais avoir davantage de temps maintenant pour vous apprendre quelques bêtises… Je vous embrasse tous.

Stéphanie

Curriculum Vitae

Stéphanie Prigent was born on October 10th 1975 in Chambray-les-Tours (France). In 1998, she completed her Maitrise in Cellular Biology and Physiology (Plant Biology) at the University of Toulouse. During summer 1996, she performed a stage in the Plant Physiology Laboratory (University of Tours), which aimed at better understanding the biosynthetic pathway of anti-carcinogenic alkaloids in plant cells. Afterwards, she studied the reaction of Maillard on β casein and its effects on functional properties of proteins such as emulsions properties, in the LEIMA (Laboratoire d'Etudes des Interactions des Molécules Alimentaires, INRA, Nantes). She graduated in 1999, obtaining a Doctorat d'Etudes Approfondies in Cellular Biology at the University of Nantes. In September 1999, she started her PhD thesis in the Laboratory of Food Chemistry of Wageningen University; the results obtained are described in this thesis. From June 2005, she works as head project Junior for the French Society of Antioxidants.

The study was part of the research programme of the Graduate School VLAG (Food Technology, Agrobiotechnology, Nutrition and Health Sciences).

The following courses, meetings and conferences were attended:

Discipline specific activities

Courses:
IOP Summer School of Industrial Proteins (Utrecht, 2000)
Advanced Biochemical Analysis Methods (Wageningen, 2000)
Advanced Food Analysis (Wageningen, 2002)
Statistics (Wageningen, 2002-2003)

Meetings and Conferences:
XX International Conference on Polyphenols (Munich, 2000)
3rd International Symposium on Industrial Proteins (The Hague, 2001)
4th International Symposium on Industrial Proteins (Ede, 2003)
1st International Conference on Polyphenols and Health (Vichy, 2003)
XXII International Conference on Polyphenols (Helsinki, 2004)
IOP meetings (Wageningen, 1999-2004)

Training:
Practical training on Polyphenols purification (INRA Le Rheu, 2003)

General courses and additional activities
PhD student week (Bilthoven, 1999)
Food Chemistry PhD trip (USA, 2002)
Scientific Paper Writing (Amsterdam, 2001)
Preparation PhD research proposal
Food Chemistry Seminars (Wageningen, 1999-2004)
Centre for Protein Technology discussion groups (1999-2004)

The research described in this thesis was performed at the Laboratory of Food Chemistry, Wageningen University, The Netherlands, the TNO, Zeist, The Netherlands and the Station Cidricole, INRA, Le Rheu, France. This research was supported by the Ministry of Economic Affairs trough the program IOP-Industrial Proteins (Project IIE 980019A), Senter, The Hague, The Netherlands. The study was part of the research programme of the Graduate School VLAG (Food Technology, Agrobiotechnology, Nutrition and Health Sciences).