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Covalent modification of food proteins by plant-based ingredients (polyphenols and organosulphur compounds): A commonplace reaction with novel utilization potential

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ABSTRACT

Background: Many food ingredients such as polyphenols, phenolic acids (e.g. present in fruit and vegetables) and organosulphur compounds (e.g. present in mustard, garlic and chives) covalently interact with meat, egg, dairy and plant-based proteins. The results of those interactions are manifold and range from altered technological properties (in emulsions, foams, gels) to sensory changes (colour formation, altered taste and smell) and different biological activity (allergy, antimicrobial effects, hydrolysis).

Scope and approach: The present review discusses both the positive and the negative side effects of such interactions and explores the potential to fine-tune protein functionality during processing not only in model solutions but also in more complex foods.

Key findings and conclusions: Traditionally, studies have focused on the negative effects of interactions between protein and plant ingredients (e.g. discolouration and solubility changes), but more recent studies highlight positive effects (e.g. enhanced emulsifying capacity, reduced allergy and targeted production of protein pigments). By controlling food processing conditions (e.g. protein nativity) and the food matrix (e.g. presence of antioxidative compounds or thiol groups, pH value during storage), the observed effects can be prevented or induced. On the basis of the listed findings, future processes can be developed that take such interactions into account to enable targeted co-processing of plant compounds with proteins. A better understanding of these interactions opens up a wealth of novel utilization potential.

1. Introduction

Interactions between proteins and plant molecules such as polyphenols are common and widespread in nature. The consequences can range from a strong astringency perception (polyphenol salivary protein binding) to an increased blood coagulation effect on wounds (thrombin-polyphenol interaction), which speeds up wound-healing processes (Pascal et al., 2007). Polyphenol binding to plasma and signalling proteins were found to affect different metabolic pathways in humans (Lacroix et al., 2018). These interactions also occur in food processing. For example, haze formation in juices and beer is a result of a reaction between polyphenols and proteins (Siebert, 1999). Interactions occur non-covalently as a weak and reversible association between protein and plant compounds, or occur irreversibly through covalent bonds (Rawel & Rohn, 2010). The kind of interaction depends on the plant ingredients involved and the reaction conditions. Non-covalent interactions with proteins occur with vitamins and polyphenols from fruit and vegetables (Keppler, Martin, Garamus, & Schwarz, 2015; Shpigelman, Cohen, & Livney, 2012). Due to their unstable nature, their effects on protein functionality during processing are not well predictable. Covalent interactions with proteins, on the other hand, are often observed for oxidized polyphenols, and for electrophilic secondary plant compounds such as organosulphur compounds from garlic, chives or papaya pulp. Covalent interactions between these reactive secondary plant compounds and proteins occur during storage and processing of protein-ingredient mixtures (fruit, vegetable, herbs) (Kuhn, von Oesen, Hanschen, & Rohn, 2018a; Rawel, Huschek, Sagu, & Homann, 2019) or during extraction of plant-based proteins (Karefyllakis, Salakou, Bitter, van der Goot, & Nikiforidis, 2018). Covalent interactions are often seen negatively because they

Abbreviations: AITC, allyl isothiocyanate; BLG, β-lactoglobulin; CLA, chlorogenic acid; DADS, diallyl disulphide; EGCG, epigallocatechin gallate; ITC, isothiocyanate; PPO, polyphenoloxidase

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alter the expected physico-chemical properties of proteins. For example, irreversible binding often changes protein solubility (Keppler et al., 2017) and is reported to reduce the bioavailability of essential amino groups. But it is of an advantage with respect to inhibiting microbial degradation of proteins. The latter effect relevant in animal feed as the amount of protein digested in the rumen is reduced, whereas the amount available in the small intestine is increased (Mueller-Harvey, 2006). Compared with non-covalent modifications, the covalent modification can alter protein functionality irreversibly. For this reason, a closer look at such interactions is particularly interesting.

An approach to avoid the covalent interactions described above is to remove these plant molecules before further use of the material. Unfortunately, such tactics are not often successful and require a lot of resources (energy and chemicals). A better approach is therefore to control these interactions in such a manner that they become a way to refine the functionality of proteins during processing. So far, only a few comprehensive overviews describe the range of functional properties derived from covalent protein modifications through polyphenols: Ozdal, Capanoglu, and Altay (2013) and Quan, Benjakul, Sae-leaw, Balange, and Maqsood (2019) have summarized changes in functionalities derived from polyphenol binding to proteins with respect to antioxidative and emulsifying capacity in final products; Liu, Ma, Gao, and McClements (2017a) reported possible applications such as nutraceutical delivery systems in combination with polysaccharides, and Bittner (2006) focused on chemical, sensory and biological consequences. The potential of other bioactive molecules has not yet been described.

Here, we provide an overview of the possible advantages and disadvantages of protein modifications due to covalent reactions with plant metabolites relevant to food processing (polyphenols as well as sulphur compounds) and identify the process conditions under which they can be prevented or induced. On the basis of the findings, processes can be developed in the future that use those interactions to co-process plant compounds with proteins. Therefore, this work is divided into four parts. The first part summarizes the covalent reactions of purified proteins in well-defined matrices with frequently occurring and reactive plant compounds. These include polyphenols and isothiocyanates and allyl sulphides, classes of organic sulphur compounds. The second part assesses how these modifications affect the physicochemical and functional properties of different food proteins. The third part gives a brief discussion on the biological and nutritional effects, ranging from allergy and antimicrobial effects to hydrolysis pattern. The fourth part provides an outlook on the consequences of processing real food matrices in which all ingredients are present naturally. This section also describes how it is possible to use and control reactions/modifications between proteins and plant molecules.

2. Part I: binding reactions

2.1. Polyphenols

Detailed reviews on the covalent interaction mechanism between proteins and polyphenols have been published (Kroll, Rawel, & Rohn, 2003; Perez-Gregorio & Simal-Gandara, 2017; Rohn, 2014; Schieber, 2018). Therefore, this section contains only a brief overview. Polyphenols can be oxidized enzymatically (e.g. catalysed by polyphenol oxidases [PPO]) or by autoxidation (e.g. in an alkaline environment or in the presence of oxidizing agents) to electrophilic *o*-quinones, which can undergo various consecutive condensation reactions with other *o*-quinones (i.e. dark melanin pigments) and/or bind to proteins covalently (Fig. 1A). Susceptible amino acid side chains are free thiol groups (i.e. cysteine side chains = S adducts) and amino groups (i.e. alpha and epsilon amino groups = N adducts). Less reactive side chains include indole groups (i.e. tryptophan side chains), amide groups (i.e. asparagine and glutamine side chains), imidazole groups (i.e. histidine side chains) and the phenolic side chain of tyrosine (Bittner, 2006; Li, Jongberg, Andersen, Davies, & Lund, 2016; Prigent, Voragen, Visser, van Koningsveld, & Gruppen, 2007). Quinones can also condensate and then modify amino acid side chains with an altered reactivity. For example, quinone-dimer condensation reactions were observed in chlorogenic acid [CLA] or caffeic acid quinones, which significantly increased their reactivity towards amino acid side-chain reactions (Prigent et al., 2008).

Curcumin is a polyphenolic compound that can covalently bind to proteins without previous oxidation due to its β-diketone moiety. Curcumin undergoes keto-enol tautomerism and is mostly in the enol form when in solution, allowing covalent binding to protein thiols (Gupta et al., 2011). Kaempferol, another polyphenol, was also suggested to bind covalently to proteins by means of keto-enol tautomerism (Kroll et al., 2003).

o-Quinone-protein adducts can be further oxidized, which leads to cross-linked protein-polyphenol aggregates (Kroll et al., 2003) (Fig. 1A). For example, the green tea polyphenols epigallocatechin gallate [EGCG] and epigallocatechin can be oxidized to a catechol-quinone with two equal nucleophilic sites on ring B, resulting in the ability of the polyphenol to crosslink proteins (Chen, Wang, Zhang, Ren, & Zeng, 2011). In addition, oxidation can occur simultaneously on rings B and D of EGCG, also leading to the cross-linking of proteins. Thus, polyphenols with higher molecular weight have a stronger propensity to form cross-links than phenolic acids, because they have several aromatic rings and thus more sites where reactions can take place. In addition to polyphenol oxidation, some amino acid residues can also be oxidized, possibly inducing protein aggregation through coupling two oxidized tyrosine residues to form an intermolecular di-tyrosine link (Prigent et al., 2007).

The pH value determines which amino acids are primarily modified: Cysteine has a lower pK_a than the amino group of lysine (8.33 versus 10.28) and reacts faster than lysine in a moderate alkaline environment where autoxidation reactions occur. In addition, the thiol moiety is also more nucleophilic than the amino group Rade-Kukic, Schmitt, & Rawel, 2011). However, only a few proteins contain a free thiol moiety (e.g. BLG or bovine serum albumin). Other amino acid residues are more abundant for binding reactions. Enzymatic oxidation occurs near the optimum pH value of the respective enzyme (e.g. PPO at neutral pH 6–7). At this pH value, thiol moiety reactions are slower than at alkaline pH. No significant differences in the amino group reactivity were observed between alkaline and enzymatic modifications (Ali, Keppler, Coenye, & Schwarz, 2018; Prigent et al., 2007). Protein modifications by autoxidation were also observed at acidic pH 4, and the rate of protein modification generally increased with increasing polyphenol concentration in solution (Rawel, Kroll, & Riese, 2000).

Table 1 summarizes some recently reported covalent interactions for different food proteins with polyphenol-quinones.

Non-covalent interaction of polyphenols with proteins occurs as a reversible association mediated via hydrogen bonds, π-π interactions and non-specific hydrophobic interactions. In complex systems, both covalent and non-covalent interactions are present, and the resulting effects may overlap.

2.2. Organosulphur compounds

Allyl sulphides and isothiocyanates [ITC] are two classes of reactive organosulphur compounds that occur frequently in many vegetables and some fruits.

Allyl sulphides can be found in allium vegetables, especially garlic, onions, leeks and chives. Garlic, for example, contains approximately 33 different sulphur compounds such as alliin, allicin, ajoene, allylpropyl disulphide, diallyl trisulphide, S-allyl cysteine, vinyldithiine, S-allylmercaptocysteine (Omar & Al-Wabel, 2010). Thiosulphinates can react with free thiol groups and/or disulphide bonds of proteins, resulting in S-alk(en)ylthio-L-cysteine derivatives (Kusterer, Fritsch, & Keusgen, 2011). Similar to the reaction with polyphenols, the

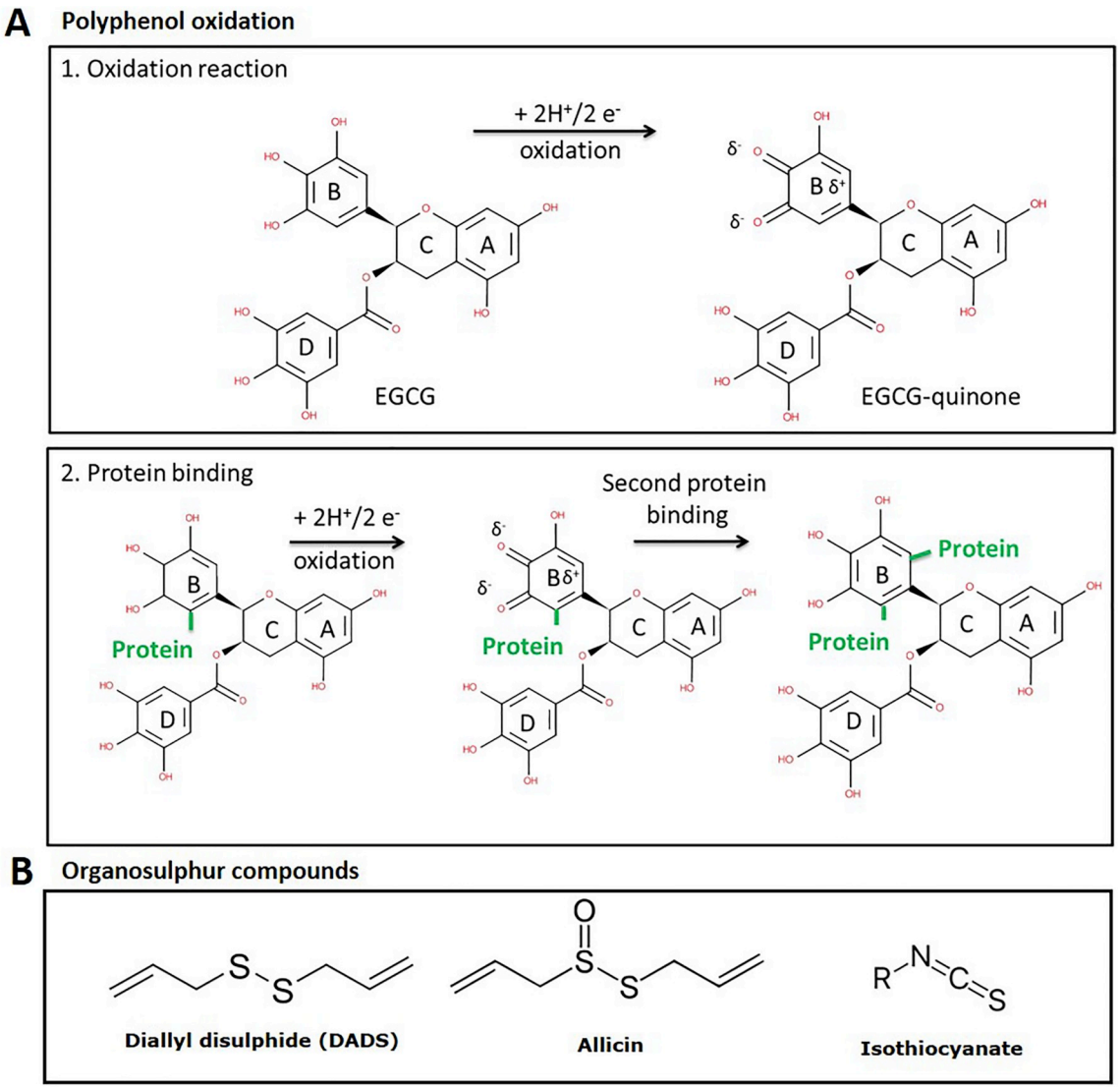

Fig. 1. A) Polyphenol oxidation: (1) Proposed simplified mechanism of epigallocatechin gallate oxidation on ring B. (2) Binding of an epigallocatechin gallate-quinone ring B to a proteins free amino or thiol group, followed by a second oxidation of EGCG at ring B. The figure was modified according to Chen et al. (2011) and Kroll et al. (2003). **B)** Organosulphur compounds: Structure of some organosulphur compounds that interact with free thiol groups of proteins and amino acids. DADS, diallyl disulphide.

Table 1
Overview of studies describing covalent binding reactions of oxidized polyphenols, phenolic acids and phenolic components with different food-derived proteins.

Protein	Phenol type	Modification type	Reference
Animal-based proteins			
β-Lactoglobulin	Epicatechin-quinone	Autoxidation (neutral pH)	Gallo et al. (2013)
Whey protein isolate	Rosmarinic acid quinone	Compare enzymatic at pH 6.5 and autoxidation (alkaline pH)	Ali et al. (2018)
Whey protein isolate	Quercetin quinone, rosmarinic acid quinone, CLA quinone	Autoxidation (alkaline pH) and enzymatic	Ali (2019)
Myoglobin	Ferulic acid, gallic acid	Autoxidation (alkaline pH)	Kroll et al. (2003)
Lysozyme, lactalbumin, bovine serum albumin	CLA quinone	Compare enzymatic at pH 5 or pH 6.0 and autoxidation (alkaline pH)	Prigent et al. (2007)
N-terminally protected amino acids	CLA quinone	Compare enzymatic at pH 7 and autoxidation (alkaline pH)	Prigent et al. (2008)
Plant-based proteins			
Sunflower proteins	CLA quinone	Autoxidation (alkaline pH)	Karefyllakis et al. (2018)
Coffee storage proteins	Caffeoylquinic acid	Enzymatic (acidic pH)	Ali et al. (2012)
Soy protein	Anthocyanin quinones	Autoxidation (alkaline pH)	Jiang et al., 2019; Sui et al., 2018
Flaxseed proteins	Flaxseed polyphenols and ferulic acid quinones	Autoxidation (alkaline pH)	Pham, Wang, Zisu, & Adhikari, 2019a, 2019b

CLA, chlorogenic acid; EGCG, (−)-epigallocatechin gallate; GCG, gallocatechin gallate.

Table 2
Overview of studies describing covalent binding reactions of sulphur containing ingredients with different food-derived proteins.

Protein	Sulphur compound	Binding sites[a]	Reference
Animal-based proteins			
BLG	Allicin	Cys	Wilde et al., 2016a, 2016c
BLG	DADS	Cys	Wilde et al., 2016a, 2016c
BSA	DADS		Negishi et al. (2002)
Vimentin	Ajoene		Kaschula et al. (2019)
-	Allicin	Cys	Zhou et al. (2008)
BSA	BITC (ground wasabi and papaya seeds)	Lys	Nakamura, Kitamoto, Osawa, and Kato (2010)
BSA	AITC	Lys	Murthy & Rao, 1979
BSA	AITC	Lys	Nakamura et al. (2009)
Insulin, BSA, ovalbumin, lysozyme	AITC (crushed *Brassica* seeds)	Lys, Arg, Cys	Kawakishi and Kaneko (1987)
Lactoglobulin	AITC	ε-Lys, α-Leu, Cys	Keppler et al., 2013, 2014a
Lactoglobulin	AITC	ε-Lys, α-Leu, Cys	Rade-Kukic et al. (2011)
Milk proteins in curd	BITC, AITC (cress)	Lys, Cys	Kuhn et al. (2018a)
Plant-based proteins			
Papain	Allicin	Cys	Miron, Listowsky, and Wilchek (2010)
Proteins in *broccoli sprouts*	Isothiocyanates	Lys, Cys	Hanschen et al. (2012)
Mustard 12 S	AITC	ε-Lys, Tyr	Murthy and Rao, 1986

AITC, allyl isothiocyanate; BITC, benzyl isothiocyanate; BLG, β-lactoglobulin; BSA, bovine serum albumin; DADS, diallyl disulphide; PITC, phenyl isothiocyanate.
[a]*Not all studies screened for all possible binding sites; often only amino and/or thiol group modifications are measured.*

organosulphur SH-conjugation reaction rate increases with increasing pH value because deprotonation of the cysteine residue favours its reactivity towards covalent modifications (Wilde, Keppler, Palani, & Schwarz, 2016a).

There are few studies on the interaction of food proteins with these sulphur compounds. Disulphide-conjugate formation can be expected to occur during food processing or food digestion (Zhang, Li, Lee, & Parkin, 2010). For example, the capturing capacity of different foods for volatile sulphur compounds (diallyl disulphide [DADS] and diallyl sulphide) was found to be high for milk (95% and 91%), as well as eggs (84% and 88%). It was suggested that capturing occurs due to thiol interactions with food components, especially with proteins (Negishi, Negishi, & Ozawa, 2002). The interaction of allicin and DADS (Fig. 1B) with BLG was described in detail recently. Both compounds reacted with the free thiol group of the whey protein at a pH value > 7 within 24 h (Table 2) (Wilde et al., 2016a, 2016c).

Cruciferous vegetables such as broccoli, cauliflower, Brussels sprouts, cress, and cabbage plants (and especially their fermentation product sauerkraut) are a rich food-based source of glucosinolates and their degradation products ITCs. Papaya pulp and seeds are another valuable source of ITCs (Nakamura et al., 2007). In 1956, ITC reactions with amino acids or proteins were used to determine the primary structure of proteins (Edman, 1956). Later, Rawel, Kroll and colleagues did extensive research on ITC food protein interactions (e.g., Kroll & Rawel, 1996; Rade-Kukic et al., 2011).

All ITCs have the R–N═C═S functional group in common. The electrophilic C atoms form thiocarbamoyl adducts with thiols or thiourea adducts with amines (Nakamura, Kawai, Kitamoto, Osawa, & Kato, 2009). Besides cysteines and amino groups (Hanschen et al., 2012; Nakamura et al., 2009; Anantharamkrishnan & Reineccius, 2020), tryptophan, methionine and tyrosine were also found to be potential targets of ITCs (Kroll & Rawel, 1996; Murthy and Rao, 1986). Further, reactions between ITCs and other nucleophilic agents such as β-dicarbonyls and carboxylic acids are suggested (Zhang, Wade, Prestera, & Talalay, 1996). Similar to these reactions for polyphenols and allyl sulphides, the rate of the ITC conjugation reaction is dependent on pH, ITC concentration and available binding sites (Hanschen et al., 2012). In addition, thiol reactions are potentially unstable, especially at higher pH values at which cysteine exchange reactions occur (Kuhn et al., 2018b). Therefore, the bound molecule can be liberated and then irreversibly modify an amino group (Kuhn et al., 2018b; Nakamura et al., 2009).

In addition, the allyl isothiocyanate [AITC] reaction with specific amino acid residues of BLG was found to be controllable by changing the protein/ITC ratio (Keppler et al., 2013; Keppler, Koudelka, Palani, Tholey, & Schwarz, 2014a). Binding of AITC was also found to be dependent on the primary structure of the proteins (local changes in the pK_a value of the amino acid residue), and on the tertiary and quaternary structure (accessibility of the binding site of the protein as well as the size of the ITC) (Cejpek, Valusek, & Velisek, 2000) (Table 2).

3. Part II: Influence on protein functionality in controlled systems

In controlled systems, a limited number of reactants are incubated under defined reaction conditions so that the effects on protein functionality can be traced back. Covalent and non-covalent interactions occur simultaneously, even in model systems, although a high pH, long incubation time or the presence of enzymes shifts the equilibrium towards more covalent modification. In model systems, unbound molecules are often removed by dialysis, ultrafiltration or evaporation of the volatile sulphur compounds. These techniques can also be used to decrease the amount of non-covalently bound molecules. However, the extent of protein modification varies depending on the modification conditions or the protein used for modification. The results listed here reflect only an average of different observations.

3.1. Interfaces: air-water and oil-water interfaces and antioxidative capacity

Polyphenols have a predominantly positive influence on the physical and chemical emulsion stability of proteins through covalent interaction.

Addition of covalent CLA quinone to whey proteins resulted in a more open protein structure, thereby enhancing the emulsifying and foaming properties (Xu et al., 2019). Other studies identified an altered hydrophilic-hydrophobic balance in modified proteins as the cause of the enhanced emulsifying properties. For example, gelatine covalently modified with oxidized tannic acid, caffeic acid or ferulic acid resulted in a lower hydrophobicity of the adduct, which increased the surface activity in emulsions and lowered the size of emulsion oil droplet sizes (Aewsiri et al., 2009). Several other studies confirmed an overall enhanced emulsifying capacity of modified proteins; for example, a covalent modification of BLG with CLA increased the creaming stability of medium-chain triglyceride-oil emulsions (Ali, Homann, Khalil, Kruse, & Rawel, 2013). Similar effects were reported for protein-bound oxidized quercetin and covalent hydroxycinnamate-protein interactions (Rawel & Rohn, 2010). Covalent adducts of lactoferrin with CLA or EGCG quinone also exhibited increased physical stability of β-carotene

bilayer emulsions under freeze-thaw, ionic strength and thermal treatments (Liu, Wang, Sun, McClements, & Gao, 2016a). In addition, covalent adducts of α-lactalbumin with EGCG or CLA showed enhanced creaming stability (Liu et al., 2016a).

With respect to chemical stability, a positive effect on the antioxidative capacity was reported for gelatine-tannic acid, gelatine-caffeic acid and gelatine-ferulic acid adducts (Aewsiri et al., 2009), lactoferrin-EGCG adducts (Liu et al., 2016a) and for BLG-CLA adducts (Ali et al., 2013). Therefore, those studies suggested that these modifications could be useful to prevent lipid oxidation reactions in emulsions. Likewise, a direct comparison of the surface activity and antioxidative capacity revealed that covalent adducts of proteins were superior to non-covalent complexes (Wei, Yang, Fan, Yuan, & Gao, 2015).

In comparison with most phenols, organosulphur compounds are more electrophilic in nature and are much smaller (~100 Da instead of ~400 Da), which makes their accessibility to protein binding sites easier. In addition, their water solubility is lower, which strongly increases the hydrophobicity of any protein adduct. It is likely that, depending on the number of protein-bound molecules as well as the initial hydrophobicity of the protein, such effects are not only positive. For example, the covalent modification of AITC with BLG occurred at several amino and thiol groups strongly increasing the surface hydrophobicity of the protein and resulting in a slight destabilization of emulsions at pH 7 but not at pH 4 (Rade-Kukic et al., 2011). However, the covalent modification of whey protein isolate with AITC and subsequent freeze drying resulted in protein adducts with increased surface activity and looser protein conformation at acidic pH 2 and 4 (Keppler et al., 2017). A higher backbone flexibility of the modified protein was suggested to increase the emulsifying capacity of AITC/whey protein isolate complexes at acidic pH values. In addition, increased creaming stability was observed for such AITC-BLG emulsions at pH 2–7 (Keppler & Schwarz, 2017; Keppler, Steffen-Heins, Berton-Carabin, Ropers, & Schwarz, 2018). Similar to the AITC modification of whey proteins, the modification of BLG at the free thiol group with allicin or DADS also revealed a higher hydrophobicity of the adduct, but no tests on emulsifying properties were conducted (Wilde et al., 2017; 2016a; 2016c).

An overview of several effects after modification with different plant-based compounds is given in Table 3. In summary, covalent modifications of proteins mostly exert their positive effect on the emulsifying capacity by increasing the molecular flexibility of the protein and affecting the hydrophilic-hydrophobic balance. For polyphenol modification, the increased antioxidative capacity also adds to the chemical stability in addition to an altered physical effect on the emulsion. Organosulphur compounds could be used when a lower protein surface hydrophobicity is desired (Table 4).

3.2. Gelling

The covalent modification of proteins with polyphenols was also found to increase gel strength via the reported cross-linking effects of the oxidized polyphenols. This was found for gelatine gels that were modified with rutin hydrate, caffeic acid, ferulic acid or with coffee or grape juice phenols (Strauss & Gibson, 2004). Similar findings were reported for meat gels where CLA or rosmarinic acid was covalently attached to myofibrillar protein (Cao & Xiong, 2015; Tang et al., 2017; Wang et al., 2018). Improved gelling properties (increased gel breaking force and deformation) in surimi gels were observed after covalent attachment of oxidized ferulic acid, tannic acid, catechin or caffeic acid to the fish protein. Polyphenols that induced multidentate binding were shown to be more effective than smaller phenolic acids (Balange & Benjakul, 2009).

However, several studies reported a dose-dependent effect on gelling capacity, for example, for myofibrillar proteins in meat processing. At a higher polyphenol/protein ratio than reported above, the gel deformation and breaking force decreased together with the water-holding capacity. This was caused by a significant loss of free thiol groups of proteins because of the high degree of modification with polyphenols and thereby a loss of intrinsic protein cross-linking ability. In addition, self-aggregation of the phenolic compound also disturbed homogeneous gel formation (Tang et al., 2017; Wang et al., 2018; Zhang et al., 2018). Simultaneous non-covalent interactions with increasing addition of polyphenols may also reinforce the formation of the gel structure. To the best of our knowledge, there are no studies available on the effect of organosulphur compounds on the gelling capacity of proteins. However, because most sulphur compounds bind to proteins via thiol groups, their gelling ability through covalent interactions is likely to be negatively affected.

Table 3
Overview of studies describing the functionality of covalently modified food-derived proteins.

Protein	Plant-based molecule	Functionality	Reference
Animal proteins			
Lactoferrin [LF]-dextran	CLA quinone	Ternary aggregates (polysaccharide + polyphenol + protein); increased freeze-thaw stability of emulsions; enhanced creaming stability	Liu et al., 2016a
LF-dextran	CLA quinone	Ternary aggregates	Liu, Ma, McClements, & Gao, 2016b
LF	EGCG, CLA, gallic acid quinones	Thermal stability increased; emulsifying capacity increased; pH value-dependent increase of solubility	Liu, Sun, Yang, Yuan, and Gao (2015)
Gelatine	Tannic acid, caffeic acid or ferulic acid quinones	Lower surface hydrophobicity; increased surface activity; lower emulsion oil droplet size; antioxidative stability increased	Aewsiri et al. (2009)
ALA, BLG, LF, S-Cas	EGCG quinone	Surface activity and antioxidative capacity enhanced	Wei et al. (2015)
BLG	CLA quinone	Enhanced creaming stability	Ali et al. (2013)
Whey protein	CLA quinone	Enhance emulsifying and foaming capacity; reduced allergenicity	Xu et al. (2019)
Whey protein	AITC	Emulsion stability decreased at pH 7; foam capacity increased	Rade-Kukic et al. (2011)
Whey protein	AITC	Surface activity increased at acidic pH values; molecular flexibility increased at acidic pH	Keppler et al. (2018)
Whey protein	AITC	Emulsion creaming stability was enhanced at pH 2–7	Keppler and Schwarz (2017)
Whey protein	AITC	Surface hydrophobicity decreased; significant conformational changes observed	Keppler et al. (2017)
Plant-based proteins			
SPI	EGCG	Enhanced emulsifying properties and creaming stability	Tao et al. (2018)
SPI	Anthocyanins	Improved emulsifying and foaming properties	Sui et al. (2018)
Flax seed protein isolate	Flaxseed phenols (ferulic acid)	Reduced emulsifying properties due to charge effects	Pham et al., 2019a, 2019b

ALA, α-lactalbumin; AITC, allyl isothiocyanate; BITC, benzyl isothiocyanate; BLG, β-lactoglobulin; BSA, bovine serum albumin; CLA, chlorogenic acid; LF, lactoferrin, CLA, chlorogenic acid; EGCG, epigallocatechin gallate; S-Cas, sodium caseinate; SPI, soy protein isolate.

Table 4
How to induce or prevent the interaction of protein with different plant-based ingredients. Summary[b] of reaction parameters, physico-chemical, functional and biological changes after covalent modification of proteins with plant-based ingredients.

	Polyphenols	Organosulphur	
		Allyl sulphides	Isothiocyanates
Reaction parameters			
Primary modification site	S and N	S	S and N
Stability	Irreversible	S binding reversible at alkaline pH	
Prerequisite	Oxidation (autoxidation or catalysed by polyphenol oxidases [PPO])	Released by enzymes in crushed cell	
How to induce	pH > 7, surplus reactive plant-based molecules, higher temperature, time, any processes that unfold or hydrolyse proteins		
	Polyphenol oxidation is induced: in high moisture environment, high moisture ingredients, high moisture storage, presence of oxidants	-	
How to prevent	**Polyphenol oxidation is prevented:** by reducing agents (e.g. ascorbic acid, sulphites); PPO inactivation	**Production of reactive organosulphur compounds:** is prevented by enzyme inactivation	
	Polyphenol oxidation is slowed: at low pH values, exclusion of UV light, low oxygen levels (modified atmosphere), low temperature		
	Covalent interactions are slowed: by acidic pH values. Avoid protein denaturation/hydrolysis. Decreased reaction temperature and incubation time. Decreased plant-based molecules/protein ratio by complexing compounds with e.g. cyclodextrin or scavenge with L-cysteine as "sacrificial" molecule. Blocking of binding sites (e.g. with reducing sugars via Maillard reactions).		
Physico-chemical effects of the interaction on proteins	Protein unfolding, altered hydrophobic-hydrophilic balance, altered thermal stability	Increased molecular and thermal protein stability	Protein unfolding, altered hydrophobic-hydrophilic balance (mostly more hydrophobic)
Functional properties			
Emulsifying capacity	Enhanced	Unknown	Dependent on pH
Gelling capacity	**Low modification level:** enhanced by quinone-protein-protein and protein-quinone-protein cross-links	Blocking of thiol groups likely hinders protein-protein interactions	
	High modification level: blocking of thiol groups hinders protein-protein interactions		
Colour formation	Yes	No	
Sensory perception	Depends: part of flavour formation, but also off-flavour possible	Improved: initial sulphur taste and smell reduced	
Antioxidative capacity	Higher than single components	Unlikely but unknown	
Biological effects			
Antimicrobial activity	Weak or not detected	Unknown	No longer evident
Allergenicity	Reduced	Unknown	Unknown
Hydrolysis	Enhanced, but longer peptides because of steric hindrance	Unknown, but unlikely	Longer peptides because of steric hindrance
Likelihood of occurring during processing	Alkaline extraction of plant-based proteins. Fermentation and roasting of cocoa and coffee beans. Heating or storage of honey. Storage of commercial milk-cocoa drinks. Air drying of tobacco leaves. High moisture ingredients/storage, alkaline ingredients	Alkaline ingredients, alkaline extraction of plant-based proteins. Possible to occur in salad dressing with herbs, in sausages or eggs with mustard, garlic or chives, herbal curd during > 4 h storage.	
Possible application	Functional antioxidant in emulsions, foams and gels. Texturizing capacity due to cross-linking with polyphenols/ replacer of gluten, green colorant. Blocking of protein binding sites to modulate Maillard reactions	Enhanced bioactivity of volatile sulphur compounds?	Enhanced emulsion creaming stability. Blocking of protein binding sites to modulate Maillard reactions

[b]The effects reflect most of the findings so far; deviations are possible depending on the properties of the protein and the reacting plant-based molecule. N, amino group binding site; PPO, polyphenol oxidase; S, thiol group binding site.

3.3. Colour formation

The polyphenol-quinone reaction with amino acid residues can result in different coloured complexes ranging from deep brown, yellow, to green (Bongartz et al., 2016; Iacomino et al., 2017). The resulting colour can often be related to the preceding reaction. Condensation reactions between unbound quinones in the absence of amino acid residues lead to a dark brown colour. Reaction of quinone with thiol groups of amino acid residues results in discolouration, and light brown to yellow colours can emerge as a mixture of brown quinone condensation products in solution together with thiol protein-bound quinones (Pierpoint, 1969). More recent studies showed that green colouration, as frequently observed for CLA in sunflower protein extracts (Wildermuth, Young, & Were, 2016), are caused by semiquinone-type radicals, which lead to dimer formation, cyclization and further reactions with amino groups to the green coloured benz acridines (Namiki, Yabuta, Koizumi, & Yano, 2001; (Bongartz et al., 2016)). Green and yellow colours resulted from a semi-quinone radical of alkaline oxidized CLA, whereas orange and brown coloured proteins occurred in the presence of PPO at neutral pH (Ali et al., 2012). In addition to the binding site and the oxidation mode (autoxidation or PPO mediated), the quinone itself influences the final colour: CLA quinone resulted in green protein solutions, and quercetin-, and catechin-quinones led to dark orange and light brown solutions, respectively (Seczyk, Swieca, Kapusta, & Gawlik-Dziki, 2019).

Colour intensity might be reduced by inducing more thiol than amino group interactions with the oxidized polyphenols (Li et al., 2016). Black/dark brown discolouration is excluded by adding reducing agents such as ascorbic acid or by removing unbound quinone (Saltveit, 2018). The addition of polyphenol scavenging or complexing agents such as β-cyclodextrin was also found to reduce protein-polyphenol color formation successfully (Zhang et al., 2018) (Table 4).

As a positive outcome, these coloured complexes were even suggested for use as natural green pigments in food colouration, because natural green pigments besides chlorophyll are scarce (Iacomino et al., 2017; Schieber, 2018). Inducing strong green colour formation could be possible in alkaline conditions, because protein modifications and polyphenol oxidation are favoured (Table 4). Other conditions that promote green color formation in foods are storage at relatively high humidity as well as the use of high moisture ingredients (Liang & Were, 2018; Rogers, Hahn, Pham, & Were, 2018).

There are no reports on colour changes of proteins after interaction with organosulphur compounds.

3.4. Taste and smell

It can be assumed that the bitter taste of most polyphenols is masked by binding it to a protein, because it might no longer be detectable by receptors. This reaction could also be part of aroma development during fermentation, roasting and drying of tea, cacao, coffee and tobacco (Rawel et al., 2019). Of course, off flavours can be formed as, for example, in oxidized wines (Bittner, 2006). Accordingly, the aroma strongly depends on the ingredient mixture and the amount of condensed quinones.

For organosulphur compounds, which are pungent and volatile, a positive masking effect was observed after protein interaction. Allicin-protein complexes can be added to a cold coffee drink without negative sensory effects (Wilde, Keppler, Palani, & Schwarz, 2016b). The deodorization of sulphur compounds (e.g. DADS) in the breath after consumption of allium vegetables was also suggested to be most effective with milk or isolated bovine serum albumin (Negishi et al., 2002). This effect can be used positively to mask unwanted flavour, but other flavour bindings (such as aldehydes) to proteins can also reduce the desired aroma effects in foods (Meynier, Rampon, Dalgalarrondo, & Genot, 2004).

3.5. Mouth feel and food texture

Protein aggregation on a mesoscopic level can result in various food textures on a macroscopic level such as gel formation (see section on gelling). In addition, aggregation at a microscopic level may result in particle formation on a length scale of several micrometres with an impact on the mouth feel. Whey protein particle formation is a well-known effect used in dairy production to substitute the creamy mouth feel of fat droplets. The particle formation depends on a combination of heat- and shear stress processes. Because protein cross-linking and gelling can be positively influenced by addition of covalent (but also non-covalent) bound polyphenols, the controlled interaction of protein-polyphenol aggregation may open-up new strategies for the development of particle processing routes (see part IV).

Another application for covalent protein modifications is to prevent hardening of the food texture. This is a phenomenon relevant for foods with a high protein content (e.g. protein-based bars) in the presence of reducing sugar. The accumulation of advanced glycation end-products occurs by glycation reaction sequences. These glycation-based adducts induce instability during the storage of protein-rich food, resulting in browning products and hardening (Purwanti, van der Goot, Boom, & Vereijken, 2010). The binding of phenolic compounds to lysyl residues of the protein blocks the glycation reaction and prevents hardening of protein-based bars. The effect was verified for cyanidin 3-O-rutinoside and quercetin 3-O-rutinoside with whey proteins via H-bonding and π-π interactions (Khalifa et al., 2019) and can be expected to be similar for covalent binding reactions with lysyl residues. Blocking of amino groups by covalent modification with polyphenols is a promising strategy to modulate Maillard reactions in foods (Lund & Ray, 2017; Troise et al., 2014). A similar approach might be possible with amino group-binding sulphur compounds such as some ITCs.

4. Part III: Overview of biological effects

4.1. Bioavailability and hydrolysis

Covalent attachment of polyphenol-quinones or phenolic acid quinones to protein amino groups was found to result in an increased rate of hydrolysis mediated by partial protein unfolding due to modification (Jiang et al., 2019). At the same time, longer peptide residues were obtained after protein hydrolysis because the protein modification sterically blocks cleavage sites for the enzymes (Cirkovic Velickovic & Stanic-Vucinic, 2018). Thus, some losses of essential amino groups such as lysine are likely. Loss of essential amino acids was also reported after covalent attachment of organosulphur compounds to proteins. Covalent modification of free amino groups in egg white protein with ITC reduced the availability of lysine significantly in animals (Hernández-Triana, 1996). Likewise, AITC binding to BLG through lysine residues altered the tryptic and chymotryptic digestion of the modified protein, resulting in blocked cleavage sites and an altered peptide pattern (Keppler et al., 2014a).

Further, trypsin digestion was affected by AITC modification of mustard 12S protein, although papain and α-chymotrypsin showed no altered cleavage (Murthy and Rao, 1986).

With respect to thiol group binding of organosulphur compounds, disulphide linkages via protein thiol groups are unstable, and it is likely that bound molecules can be liberated during digestion (Kuhn et al., 2018b). Thus, both the amino acid value of the protein and the bioactivity of the bound molecule might not be negatively affected in that case. Likewise, the bioavailability of allicin covalently bound to cysteine in BLG is reported not to be reduced (Wilde et al., 2016b). BLG-allicin modification was even suggested to have the potential to fortify foods with bioactive garlic compounds, because the protein reduces its pungent smell while stabilizing the volatile organosulphur compound without decreasing its bioavailability (Wilde et al., 2017).

In conclusion, protein modification to amino groups can lead to loss of essential amino acid residues and changes in the hydrolysis pattern, and the hydrolysis rate can be increased due to protein unfolding. For thiol modifications with organosulphur compounds, enhanced bioavailability is possible due to the reversible nature of this binding site (Table 4).

4.2. Antimicrobial effects

Low antimicrobial effects on gram-positive *Staphylococcus aureus* (LMG 10147 and Mu 50) were observed for rosmarinic acid quinones that were covalently bound to BLG via PPO: In contrast, unmodified or alkaline-induced protein modification with rosmarinic acid showed no significant effect on the bacteria (Ali et al., 2018). It was speculated that polymerized quinones induced by PPO could form melanin, which were reported to have antimicrobial activity on *Bacillus cereus*, *B. subtilis*, *Micrococcus luteus* and *S. aureus*. Another possibility is the oxidation of tyrosine residues by PPO to l-3,4-dihydroxyphenylalanine [L-DOPA], which could exert antimicrobial activity (Zhao, Li, Wang, & Jiang, 2007). Conjugates between lysozyme and avarone, a sesquiterpene quinone of marine origin, were found to exert antibacterial activity against a broad range of bacteria. Here, both compounds already have antimicrobial activity, and it was proposed that when modified lysozyme is hydrolysed by bacterial defence enzymes, the emerging peptides with the quinone bound can still have an antimicrobial effect (Novakovic, Andelkovic, Zlatovic, Gasic, & Sladic, 2012).

For organosulphur compounds such as ITCs, which are known for their strong antimicrobial effect when unbound, it was shown that this biological activity was lost after covalent binding to BLG. This was expected, because the antimicrobial activity of ITC is mediated by binding to bacterial proteins. When ITC is already protein bound, it can no longer interact with the bacterial proteins (Keppler et al., 2017).

4.3. Allergy

The most common type of allergy is immunoglobulin E (IgE)-associated allergy, mediated by IgE binding to surface patches on proteins resulting in cross-links on mast cell and basophil surfaces (Plundrich et al., 2014). IgE binding epitopes can be linear stretches (consisting of 8–10 specific amino acids in a line) or conformational (specific secondary and tertiary structure elements). Some linear epitopes are also present in conformational epitopes, because the secondary and tertiary conformation results in the convergence of different amino acids that can form a pattern that resembles those of the linear epitopes (Li, Yuan, He, Gao, & Chen, 2015).

Covalent modification of ovalbumin with CLA quinone resulted in reduced allergenicity *in vitro* (Lu et al., 2018) and similarly, the allergenic potential of whey protein BLG was reduced after covalent modification with EGCG and CLA *in vitro* (Wu et al., 2018; Xu et al., 2019). Covalent modification of peanut proteins by quercetin (aglycone or glycoside form) from cranberry or blueberry (Plundrich, Bansode, Foegeding, Williams, & Lila, 2017) also resulted in decreased IgE recognition. To the best of our knowledge, no studies have been conducted on the allergenicity of proteins after covalent modification with organosulphur compounds.

The reasons for the altered allergenicity are likely conformational changes that also affect epitopes and/or steric blocking of IgE binding sites. Further studies are needed to elucidate if this effect is only evident *in vitro* or is also seen *in vivo*, but the use of polyphenols to reduce the allergenic potential of proteins is certainly an interesting route for further exploration (Table 4).

5. Part IV: Evaluation of modifications during processing and extraction

Most of the reactions between proteins and plant constituents described above have been studied in models or controlled systems. These interactions are also expected to occur during food processing, i.e. extraction, fermentation, roasting, crushing and mixing of ingredients and complete foods. There are not many studies on this topic, however, covalent protein modifications with ingredients occur constantly during processing, and a closer look at these reactions in real foods could provide valuable insights on how to either prevent or use such interactions in food processing.

5.1. Effect of food properties on modifications

Although covalent protein modifications can be observed in foods with acidic to neutral pH values, they are generally slowed down by acidic pH values:

Covalent interaction of milk proteins with polyphenols was observed in commercial chocolate milk drinks after 4 h at room temperature, although under the given conditions (neutral pH value, short incubation time, protein/polyphenolic ratio), only a small proportion of the proteins were covalently altered and the loss of essential amino acids was therefore minor (Gallo, Vinci, Graziani, Simone, & Ferranti, 2013). However, protein modifications can also occur in plants, as was reported for covalent protein modification in broccoli sprouts with endogenous ITCs (Hanschen et al., 2012) as well as in processed foods, as reported for the interaction between ITCs from chopped garden cress and milk proteins in slightly acidic herbal curd or pH neutral milk (Kuhn et al., 2018a). Many covalent protein modifications were also observed in meat (acidic to neutral pH), probably because addition of rosmarine extracts or other polyphenol rich seasonings as antioxidants is a common practice (Jongberg, Gislason, Lund, Skibsted, & Waterhouse, 2011; Tang et al., 2017; Wang et al., 2018; Zhang et al., 2018). However, sunflower butter cookies made with acid ingredients (such as sour cream, buttermilk, yoghurt or honey; all pH values < 4.8) or acidifiers had a lower covalent protein modification with oxidized chlorogenic acid and thus a lower green colour intensity than biscuits made from neutral maple syrup or alkaline baking powder (Liang, Tran, & Were, 2018).

Thus, the pH of the ingredients can be used to reduce covalent modifications, but also to induce them: Egg proteins are reportedly prone to covalent interactions with polyphenols or organosulphur compounds during processing because of their inherent alkaline pH value (Hanschen et al., 2012). In addition, alkaline protein extraction from plant materials induces protein modification (Karefyllakis et al., 2018; Karefyllakis, van der Goot, & Nikiforidis, 2019b; Wildermuth et al., 2016). An interesting route to generate functional modified extracts was suggested previously. By adding defined, highly reactive small cysteine-containing molecules to plant waste material in alkaline conditions, fast and preferential binding to them was induced. These modified extracts from plant by-products had a high antioxidative capacity and can be mixed as ingredients to other foods (Selga and Torres, 2005).

Not only the pH value but also the composition of the rest of the food matrix determines if the protein modification is induced or hindered. Excess secondary plant compounds in relation to proteins may have negative or uncontrollable effects because the plant compound:-protein ratio determines the grade of protein modification. The reason is that non-covalent modifications occur increasingly besides the covalent bonding; another reason is the increasing extent of protein modification, which can result in complete blocking of all protein binding sites. For example, for meat gels (see section on gelling 3.2), the addition of polyphenol-quinone was found beneficial at a biological (antioxidative capacity) (Jongberg et al., 2011) as well as functional level (gel strength) (Tang et al., 2017). However, surpassing a certain level of protein thiol modification then reversed the positive functional effects again (Tang et al., 2017; Wang et al., 2018; Zhang et al., 2018).

A measure of control on these interactions is possible by inducing specific protein modifications with enzymes: For example, protein-polysaccharide networks can be achieved via enzymatic oxidation of phenolic moieties such as tyrosine on proteins and esterified phenolic acids in polysaccharides (e.g. ferulic acid in arabinoxylan) (Selinheimo, Lampila, Mattinen, & Buchert, 2008). In wheat bread production, enzymatic oxidative cross-linking of phenolic compounds can be controlled with enzyme type (laccase, tyrosinase) as well as with the substrate concentration (e.g. arabinoxylan) and can lead to improved bread quality. Such cross-linkings were found to strengthen the gluten network in bread, thereby increasing the effective gluten concentration (Selinheimo, Autio, Kruus, & Buchert, 2007).

There are other foods where, for example, a combination of ingredients affects covalent modification, such as honey. Maillard reactions lead to sugar-protein complexes that also contain and likely co-induce covalently protein-bound polyphenol-quinones (Brudzynski, Sjaarda, & Maldonado-Alvarez, 2013). There are also reports on modulating or preventing Maillard reactions during thermal processing of milk by covalently blocking protein binding sites with polyphenols (Troise et al., 2014).

A similar competition for binding sites as in the Maillard reaction with reducing sugars arises when oxidized polyphenols, organosulphur compounds and proteins are present together. *o*-Quinones can not only react with proteins or with themselves but can also covalently bind organosulphur compounds (Negishi et al., 2002). It gets even more complex when the interaction of flavour molecules (Anantharamkrishnan & Reineccius, 2020) or secondary lipid oxidation compounds (aldehydes) with proteins (Meissner, Keppler, Stöckmann, Schrader, & Schwarz, 2019) are also considered, because these also compete for free amino and thiol groups.

Blocking of protein binding sites with reducing sugars or with aldehydes is a possible route to prevent interactions with polyphenols or organosulphur compounds. Another way to prevent or even reverse the covalent polyphenol-protein interaction is the presence of reducing agents such as ascorbic acid in foods (Landi, Degl'Innocenti,

Guglielminetti, & Guidi, 2013) (Table 4). Those reducing agents, however, are not expected to affect the interaction of organosulphur compounds (e.g. allicin, DADS) with proteins.

Thus, one problem in predicting the outcome of modifications is the complexity and rich composition of the matrices due to the presence of several different protein and plant compound classes (ingredients), all of which behave differently.

5.2. Effect of food processing on modifications

The interaction of ingredients is strongly influenced by the processing of food. The choice of processing parameters therefore makes it possible to control the degree of protein modification by covalent interaction within certain limits. The more complex the food matrix and the processes, the more difficult it is to trace individual reactions and therefore to predict functionality changes. Increased temperatures, as they occur during roasting or cooking, can inactivate PPO and thus inhibit enzymatic browning (Iqbal et al., 2018), but they can also degrade polyphenols to quinones via autoxidation in liquid solution (Wang, Zhou, & Jiang, 2008). For example the preparation of traditional Karak Chai tea entails prolonged heating of milk and tea-polyphenols, which probably promotes covalent interactions.

Organosulphur compounds also degrade during heat treatment, but many of the resulting reaction products of polysulphides can still interact with amino acid residues (Rao et al., 2015). Processes that denature or hydrolyse proteins often increase the number of available protein binding sites and their accessibility for covalent (but also non-covalent) modifications. On the other hand, process-induced protein aggregation can in turn impair the accessibility of binding sites.

It is also worthwhile studying traditional techniques of food processing with respect to covalent protein modifications to better understand their effects. Processes that entail prolonged storage or incubation and drying in the presence of oxygen and/or PPO result in high polyphenol oxidation (e.g. fermentation of tea, sun drying of tobacco) (Bittner, 2006). In addition, also high humidity processing (such as fermentation) can accelerate polyphenol oxidation and thus protein modifications (see the section on colour formation). Specifically, the combination of fermentation and roasting (e.g. of cocoa (Rawel et al., 2019) or coffee beans (Ali et al., 2012)) not only induces Maillard reactions but almost certainly also quinone-amino acid interactions. The formed complexes are also part of the development of aroma and texture appreciated by consumers (Rawel et al., 2019). Other traditional techniques involving the processing/fermentation of soy, such as the production of tempeh, may involve protein modifications with both sulphur compounds and oxidized polyphenols. Soya contains not only a high protein concentration but also both classes of substances. To the best of our knowledge, there are few studies on this topic. Maillard reactions, organo sulphur-compounds and polyphenol-quinones mostly compete for the same binding sites on the protein. This results also in interesting possibilities for targeted protein modifications (see the previous section and the section on mouth feel and food texture).

There is a lot of research interest nowadays in increasing the utilisation of by-products that are currently not used for human consumption. Typical examples of those products are press cakes from rapeseed and sunflower. One of the main hurdles is that the food industry is not well suited to deal with product streams containing high quantities of polyphenols. As described above, those product streams are struggling with dark colour and bitter off-taste. In addition, protein modifications cause undesirable changes in solubility. Therefore, most strategies to incease the utilisation of those by-product streams focus on removal of polyphenols or organosulphur compounds. Nevertheless, those by-product streams have great potential, and this potential can even be enhanced by controlling protein-polyphenol interactions to induce targeted functionalization of proteins during extraction or processing (Troise et al., 2014). Clearly, such an approach will strongly benefit from improved scientific insights into reactions and interactions between proteins and other plant molecules. This approach aligns with trends to focus more on functional fractions rather than highly pure protein isolates (Karefyllakis et al., 2019b; Karefyllakis, Octaviana, van der Goot, & Nikiforidis, 2019a). Such an approach will contribute to a more efficient use of plant raw materials.

6. Future outlook

In this paper, we have presented an overview of covalent interactions between proteins and plant-based ingredients, especially polyphenols and organosulphur components, and routes to induce or prevent these interactions are summarized in Table 4. It is well known that a rich variety of reactions can occur that influence the functionality of both the proteins and the ingredients. Most of those interactions and reactions are studied in model systems, but interest in studying those effects in more complex mixtures or even real food products and raw materials is increasing. Most of the research on polyphenol-protein interactions is on preventing those interactions, for example, by removing them from raw material streams that contain both proteins and polyphenols. However, controlling the interactions can be a route to tune the functionalities and possibly enhance the potential of certain underutilized products streams. In the future, more research on the effects of the food matrix and different processes on protein-plant compound interactions are needed for the targeted functionalization of food proteins. Only a holistic approach that links observed functionalities with a broad range of molecular effects will help to elucidate the important parameters and their interaction for the induction of certain functionalities, even in complex processes and matrices.

Declaration of competing interest

None.

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