

A Review of Polyphenol and Whey Protein-based Conjugates

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Proteins act as a primary food component obtained from different food sources. In contrast, polyphenols are metabolites and are abundantly present in plants, so their combination plays a crucial role in defining the functional properties of a food product. In the current review, the protein-polyphenol interactions have been briefly reviewed, along with the changes that occur because of their interaction. The mechanisms and the factors affecting the functionalities of the protein-polyphenol conjugates, e.g., the solubility, antioxidant, and gelling properties, have also been briefly reviewed. In addition, the interaction of polyphenols with whey proteins was been reviewed with various applications within the food industry, e.g., emulsifiers, foaming agents, and antioxidants. To end the review, future challenges were also highlighted.

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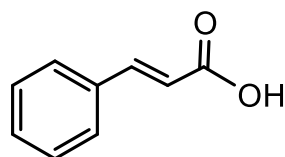
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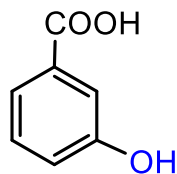
INTRODUCTION

With more than 8000 different compounds, phenolic compounds are secondary metabolites with the same structure, *i.e.*, an aromatic ring bonded to a hydroxyl group, and they are classified according to their number of carbon atoms (Kroll *et al.* 2003). Some examples of phenolic compounds are as follows: phenolic acids, flavonoids, lignans, and stilbenes in plants, which can react with protein molecules and undergo chemical change during food processing and even after consumption, as shown in Figs. 1a and 1b (Parada and Aguilera 2007; Crozier *et al.* 2009). The important factors determining polyphenol-protein interactions include the structural flexibility, molecular weight of the polyphenol, the side chain type, and the hydroxyl group number, as polyphenols with a higher molecular weight and abundant hydroxyl groups have greater protein affinity (Frazier *et al.* 2010; Xiao *et al.* 2011; Czubinki and Dwiecki 2017; Buitimea-Cantú *et al.* 2018). Protein-polyphenol interaction is classified into covalent interactions (irreversible) and noncovalent interactions (reversible), which are further reported as five groups, including electrostatic interactions, hydrogen bonds, pi (π) bonds, hydrophobic interactions, and van

der Waals (Prigent *et al.* 2003; Frazier *et al.* 2010; Rawel and Rohn 2010; McRae and Kennedy 2011).

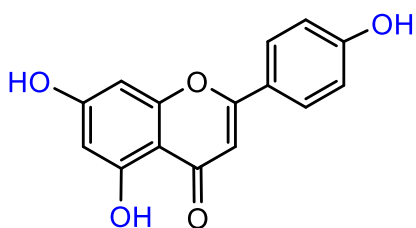


Cinnamic Acid

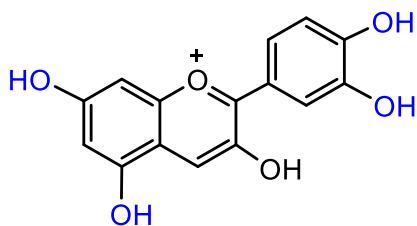
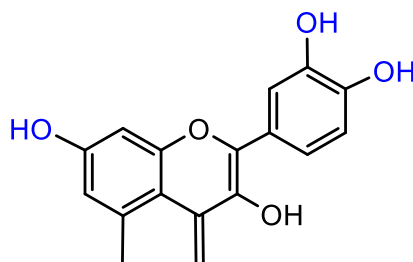


3-Hydroxybenzoic acid

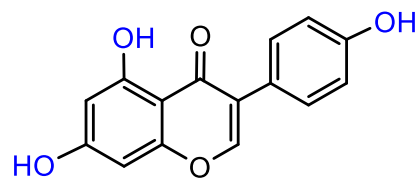
Fig. 1a. Major phenolic acid structures



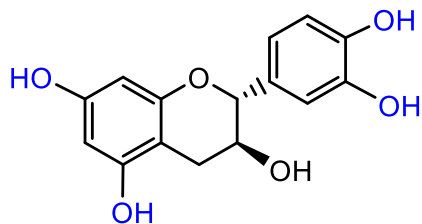
Apigenin



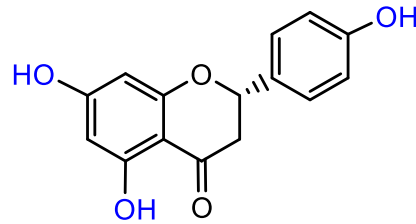
Quercetin



Genistein



Catechin



Naringenin

Fig. 1b. Major flavonoid compounds

Hydrolysis causes modification to the functional properties of proteins. However, moderate hydrolysis of whey proteins increases their heat stability as a result of the reduced secondary structure. This development does not always translate directly to more complex systems such as emulsions made using hydrolyzed whey protein, where heat stability has been shown to be adversely affected by hydrolysis of whey protein. Conjugation of proteins with polyphenol reaction has been shown to be effective in altering protein functionality (Liu *et al.* 2012; Costa *et al.* 2021). Widespread research supporting the beneficial effects of protein modification through conjugation is available in the scientific literature; improved functional properties of proteins including solubility, emulsification, encapsulation, emulsion stability, and thermal stability as a result of conjugation are well documented (Akhtar and Dickinson 2003; Buamard and Benjakul 2017, 2018).

A protein can interact through hydrophobic interactions with polyphenols. Hydrogen bonds and hydrophobic interaction are the primary noncovalent interactions regulating protein-polyphenolic interactions and involved amino acids. Examples include valine, leucine, isoleucine, alanine, phenylalanine, methionine, tryptophan, glycine, cysteine, and tyrosine. Phenolic compounds form a hydrogen bond with the protein carboxyl group, as they are hydrogen donors, so a hydrogen bond is ultimately formed between the oxygen/nitrogen molecule of amino acids and a phenolic hydroxyl group (Prigent *et al.* 2003; Rawel and Rohn 2010; Xiao *et al.* 2011; Mulaudizi *et al.* 2012; Jongberg *et al.* 2015; Tang *et al.* 2021; Xiong and Guo 2021). The mechanism of the protein-polyphenol reaction is illustrated in Fig. 2. Phenolic compounds can produce a quinone radical *via* covalent bond formation, and in the presence of oxygen and an alkaline environment, a quinone radical is generated *via* enzymatic/non-enzymatic reactions (Jongberg *et al.* 2015; Czajkowska–González *et al.* 2021; Zhao *et al.* 2021). In the second step, a quinone forms a dimer *via* a condensation reaction, known as tannins (which are brown-colored, with a high molecular weight), by reacting with the polypeptide amino acid chain *via* covalent bonding, and are re-oxidized and react with another polypeptide chain in the third step (Felton *et al.* 1989; Arts *et al.* 2001; Buchner *et al.* 2006). Examples of covalent and noncovalent protein-polyphenol interactions are explained in Table 1 (Fig. 3 and Fig. 4).

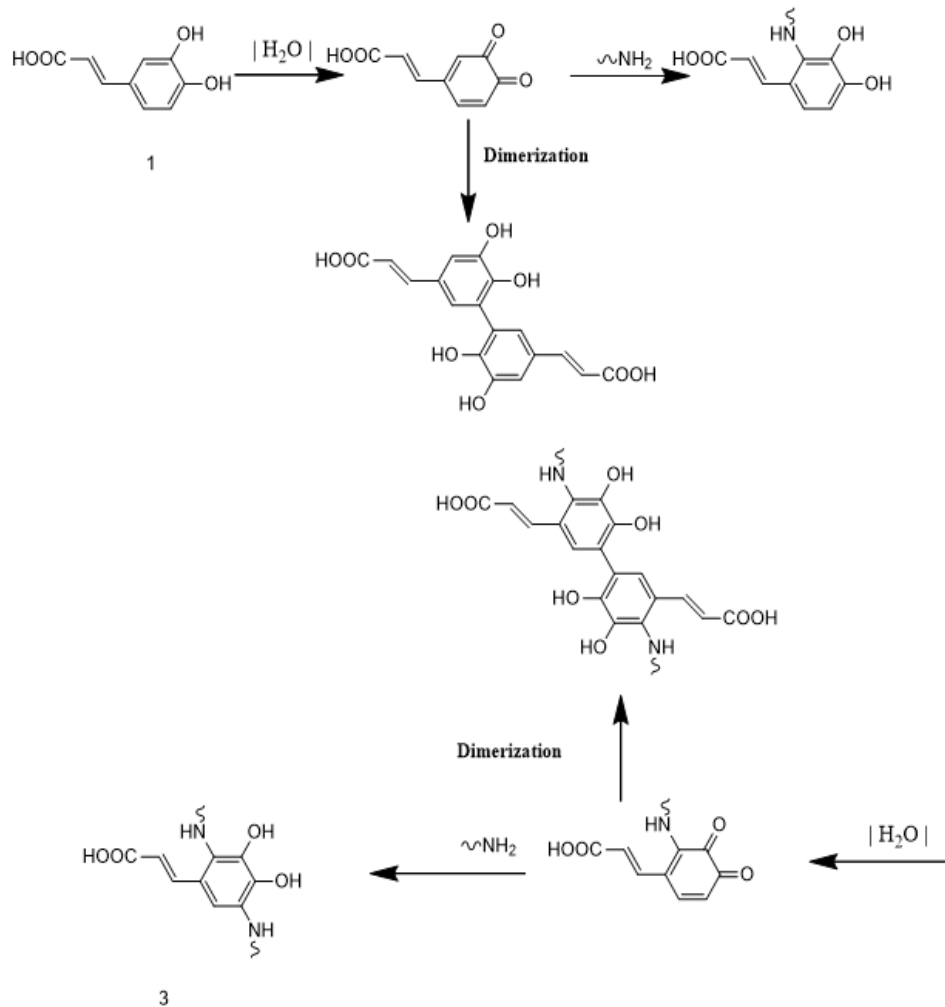


Fig. 2. Phenolic acid compound reactions with polypeptide amino acid side chains

Table 1. Covalent (Irreversible) and Non-Covalent (Reversible) Polyphenolic-Protein Interactions

Non-Covalent (Reversible) Interactions					
Noncovalent Interaction Mechanism	Phenolic Compound	Protein	Effects on Protein and/or Phenolic Compound	Assessment Method	References
Hydrogen bonding	Procyanidins of various degrees of polymerization (DP)	α -lactalbumin lysozyme, BSA	Procyanidins having medium DP may play a role in; <ul style="list-style-type: none"> • Undesirable decrease in protein solubility • Improvement in foam stability 	ITC	Prigent <i>et al.</i> (2003)
Hydrophobic binding	Walnut phenolics	Walnut proteins	Decrease in protein solubility of walnut flour obtained from whole kernels	SDS-PAGE	Labuckas <i>et al.</i> (2008)
Hydrogen bonding, Van der Waals Forces, Hydrophobic binding	Ferulic acid (-) - Epigallocatechin Green tea flavonoids (catechins)	BSA, Bovine β -lactoglobulin, β -casein in milk	Increase in protein thermal stability change of native conformation	FD and CD spectroscopy, ITC FD, CD, and FTIR spectroscopy	Wu <i>et al.</i> (2011) and Ojha <i>et al.</i> (2012)
Hydrogen binding, hydrophobic binding, and dipole-dipole interactions	Rosmarinic acid	Bovine milk whey protein (α -lactalbumin, Lactoglobulin, and Lactoferrin)	Decrease in the antioxidant potential of rosmarinic acid <ul style="list-style-type: none"> • Increase in protein stability 	Radical quenching assay (ABTS), optical density, liquid chromatography (HPLC and FPLC), DLS, and zeta-potential, FTIR and DSC	Ferraro <i>et al.</i> (2015)
Hydrophobic and hydrophilic interactions, Hydrogen bonding and hydrophobic interactions	Tea polyphenols tea, coffee, and cocoa polyphenols	Milk β -lactoglobulin B-lactoglobulin	Increase in structural stabilization of protein <ul style="list-style-type: none"> • Decrease in antioxidant activities of protein and polyphenols • Reduction in protein digestibility 	FD, CD, and FTIR spectroscopy	Kanakis <i>et al.</i> (2011)

Hydrophobic interactions	Tea polyphenols	Egg white protein; ovalbumin, and lysozyme	Conformational and second structural change of proteins <ul style="list-style-type: none"> Easier digestion of proteins at low pH; whereas reduced digestion of proteins at high pH 	FD spectroscopy and FTIR	Shen <i>et al.</i> (2014)
Hydrophobic interactions	Green tea epigallocatechin-3-gallate (EGCG)	Bovine α -lactalbumin	Increase in biological activity of EGCG	CD and FD spectroscopy, ITC	Al-Hanish <i>et al.</i> (2016)
Covalent (Reversible) Interactions					
Covalent Interaction Mechanism	Phenolic Compound	Protein	Effects on Protein and/or Phenolic Compound	Assessment Method	References
Covalent bonds (ester)	Chlorogenic-, caffeic-, and gallic acid, flavones, apigenine, kaempferol, quercetin and myricetin	Soy protein	<ul style="list-style-type: none"> Reduction in lysine, cysteine and tryptophan residues in soy proteins, The isoelectric points shifted to lower pH, Increase in molecular weight, More hydrophilic surface on soy protein, Changes in the solubility of the protein derivatives 	CD spectroscopy, DSC	Rawel <i>et al.</i> (2002)
Covalent bonds (ester and amide)	Gallic acid and rutin	Gelatin	<p>Increased thermal stability and decreased swelling (with rutin)</p> <ul style="list-style-type: none"> Modified physicochemical properties in gels treated with rutin 	Texture profile analysis, rheometry, DSC, swelling tests, scanning electron microscopy, X-ray diffraction, FTIR	Yan <i>et al.</i> (2011)
Non-disulfide covalent linkages (dityrosine bond)	Sour cherry anthocyanins	β -lactoglobulin (BLG)	<ul style="list-style-type: none"> Decreased protein allergenicity Increased protein digestibility 	SDS-PAGE, isoelectrofocusing, immunoblotting, size-exclusion and reverse-phase chromatography, mass	Tantoush <i>et al.</i> (2011)

				spectrometry, digestibility, antioxidant activity	
Covalent bonds (non-disulfide and disulfide bond)	Caffeic acid, catechin, ferulic acid and tannic acid	Fish myofibrillar protein		Texture profile analysis, color measurement, light transmission, SDS-PAGE	Prodpran <i>et al.</i> (2012)
Cross-linking	Phenolic acid, quercetin, rutin	Gelatin (Type A)	<ul style="list-style-type: none"> Enhanced mechanical strength, reduced swelling, and fewer free amino groups in gelatin gels cross-linked with phenolics Denser polymeric networks 	Free amino groups analysis, gel rigidity, swelling, dynamic light scattering	Strauss and Gibson (2004)
Covalent interactions (lutein esters)	Coffee-specific phenolics	β -lactoglobulin	<ul style="list-style-type: none"> Increased water vapor permeability Enhanced antioxidative properties of proteins Increased protein thermal stability Higher protein stability against UV light, when emulsified with lutein esters 	MALDI-TOF-MS, TEAC, Far-UV and Near-UV, CD, DSC, SDS-PAGE	Ali <i>et al.</i> (2013)
Cross-linking	Tannic acid, caffeic acid, ferulic acid	Porcine plasma protein	Increased tensile strength	Mechanical properties, water vapor permeability	Nuthong <i>et al.</i> (2009)
<p>Note: FD (fluorescence dichroism); ITC (isothermal titration calorimetry); FPLC (fast protein liquid chromatography); CD (circular dichroism); SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis); FTIR (Fourier-transform infrared); DLS (dynamic light scattering); DSC (differential scanning calorimetry); MALDI-TOF-MS (matrix-assisted laser desorption/ionization-time of flight-mass spectrometry); HPLC (high-performance liquid chromatography);, and TEAC (trolox equivalent antioxidant capacity assay)</p>					

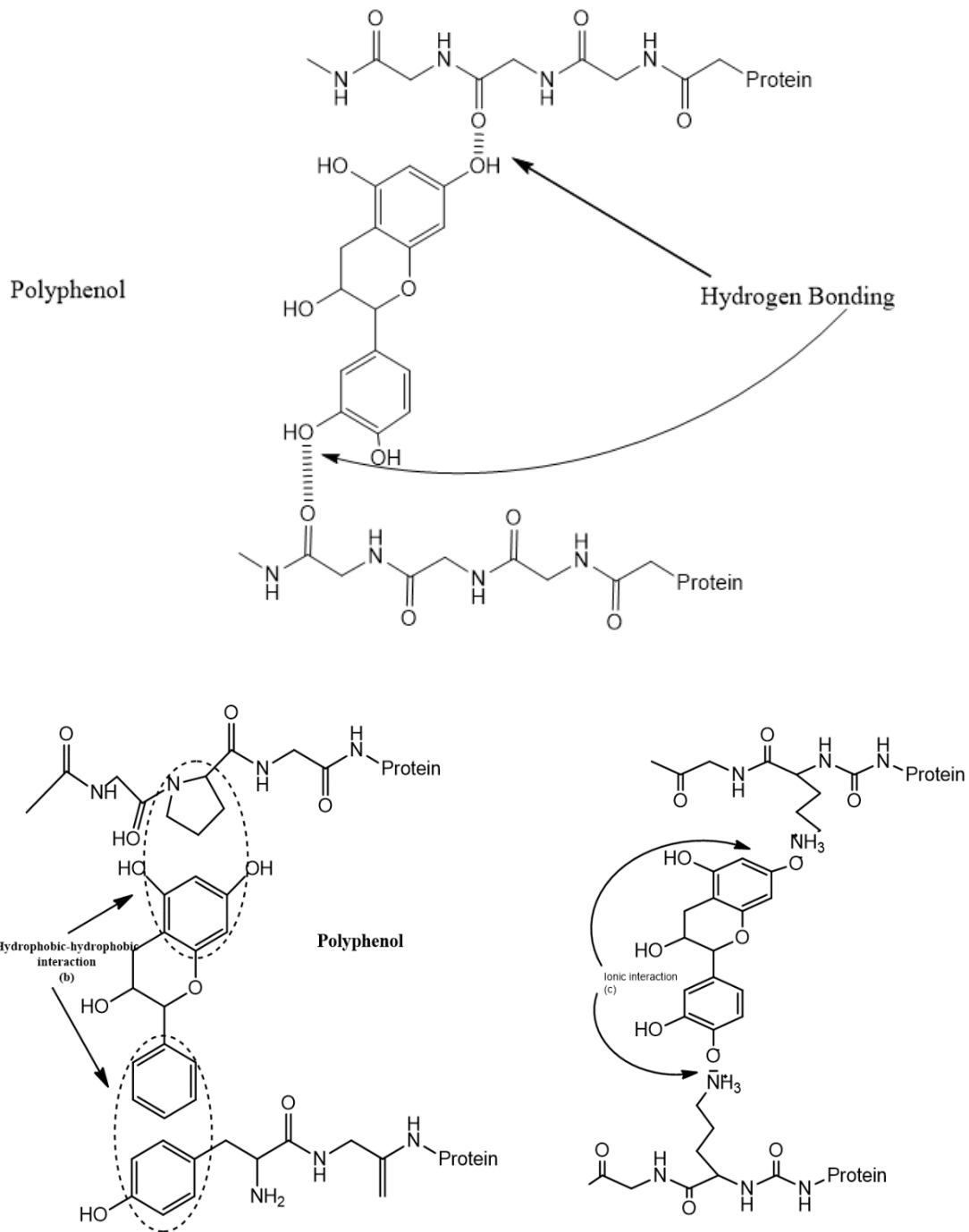


Fig. 3. Protein-polyphenol noncovalent conjugation and cross-linking of proteins *via* hydrogen bonding (a); hydrophobic-hydrophobic interaction (b); and ionic interaction (c)

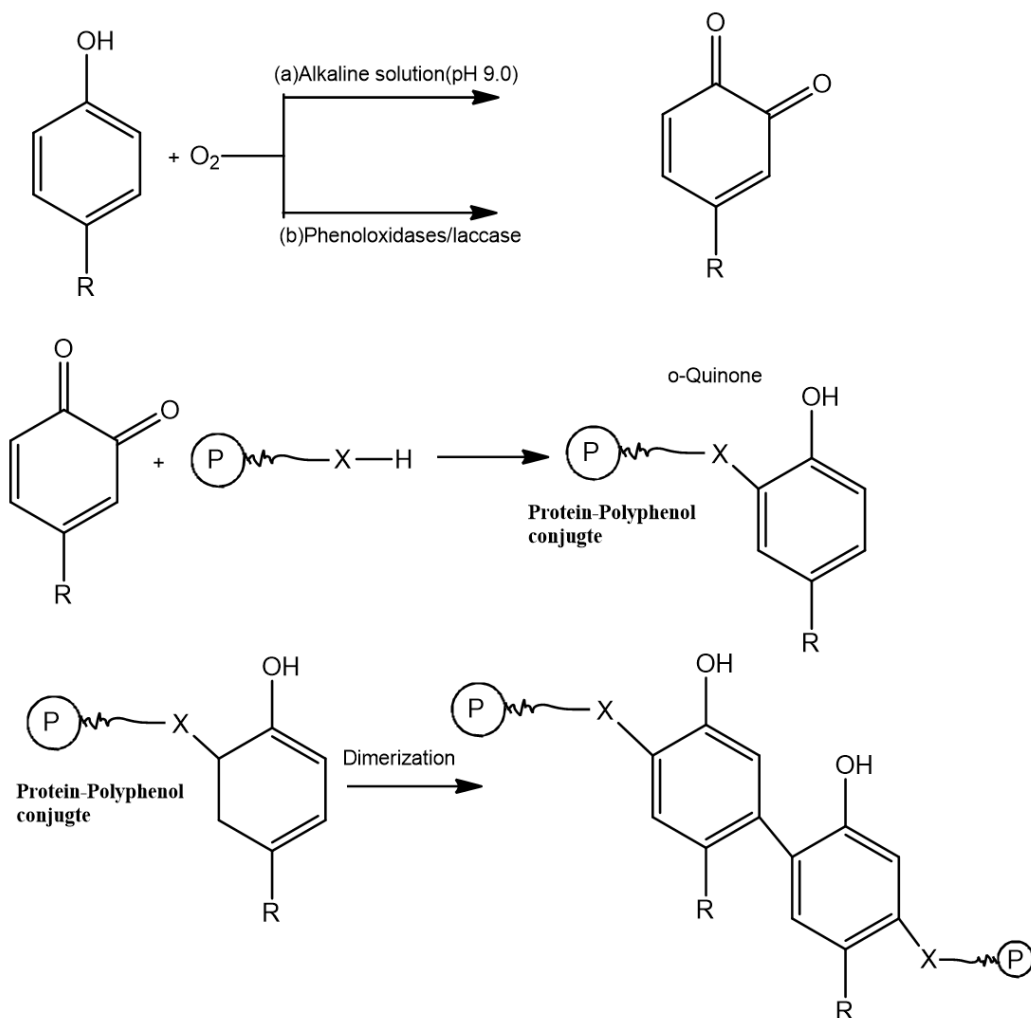


Fig. 4. Protein-polyphenol covalent conjugation and cross-linking of protein *via* alkaline (a); and enzymatic (b) reactions

FACTORS AFFECTING THE INTERACTIONS BETWEEN PROTEINS AND PHENOLIC COMPOUNDS

Many factors affect protein-polyphenol reactions, including environmental factors, *e.g.*, pH, temperature, salt concentration, and chemical presence. In addition, the protein type and phenolic compound structures are two important factors (Kroll *et al.* 2003; Ozdal *et al.* 2013; Czubinski and Dwiecki 2016) and are strongly influenced by the amino acid sequence, isoelectric point, and hydrophobicity (Frazier *et al.* 2006; Ali 2016). The higher molecular weight of protein has a higher binding affinity for phenolics, and protein size also plays a vital role in the binding affinity for proanthocyanidin (Siebert 1999; Dubeau *et al.* 2010). Phenolic compounds are different in terms of their hydroxylation degree, molecular weight, glycosylation, methylation, and hydrogenation. Temperature, an important factor that influences phenolic binding abilities for proteins, *e.g.*, 5-*O*-caffeoylquinic acid, has a decreased affinity for bovine serum albumin (BSA) and sunflower seed at a higher temperature, primarily because of hydrogen bonding (Prigent *et*

al. 2003; Guo and Xiong 2021; Guo *et al.* 2021). Another factor is pH, since additional protein-polyphenol precipitation was seen at the protein isoelectric point because of their lower solubility, showing that the optimum pH is near the isoelectric point, which varies for different proteins (Naczka *et al.* 1996, 2006; Groth *et al.* 2021). The isoelectric point of a protein is defined as the pH at which the net charge of a protein molecule is zero. Proteins are positively charged at a pH below their isoelectric point and negatively charged at a pH above their isoelectric point. The protein isoelectric point varies greatly from extremely acidic to highly alkaline values ranging from about 4.0 to 12.0. Moreover, isoelectric point values have long been used to distinguish between proteins in methods for protein isolation, separation, purification, crystallization, *etc.* (Tokamkov *et al.* 2021). Other influencing factors include the salt type, as a high salt concentration causes oligomeric protein dissociation, which causes a reduction in binding ability (Kanakakis *et al.* 2011).

Various protein functional properties, *e.g.*, emulsification, solubility, antioxidative ability, foaming ability, color, flavor, elasticity, and binding properties, could affect the quality and the sensory food property where the protein function could be affected by the phenolic compound (Abdelfatah 2013; Korpela *et al.* 2022). The protein-phenolic covalent interactions (Table 1, Fig. 4) could increase the gel network formation, including better mechanical strength and increased thermal stability, and modified proteins could be used as emulsifiers in foods to reduce the emulsion stability and increase the oil droplet size (Aewsiri *et al.* 2009). Based on the conjugates under typical conditions, it is concluded that foods properties will be affected by wide range of pH. Therefore, it might be useful for physiological saline solution.

Effects on Nutritional Properties

The nutritional value of proteins depends on various factors, *e.g.*, the amino acid composition, protein source, essential amino acids ratio, processing environment, and hydrolysis sensitivity during digestion, but it has been reported that protein digestibility and nutritional value are decreased by phenolic interactions (Rubanza *et al.* 2005; Abdelfatah 2013). Milk protein interactions with caffeic acid while heating caused a reduction in the availability of thiol groups and lysine, which is related to the presence of quinones *via* caffeic acid oxidation and the formation of an amino acid complex (Kroll *et al.* 2003). Soy protein interactions with gallic acid/caffeic acid/chlorogenic acid/quercetin cause a reduction in lysine, tryptophan, and cysteine contents. The condensed tannins, which are present in sorghum, are responsible for reduced digestibility of the sorghum protein (Emmambux and Taylor 2003).

Protein-Polyphenol Conjugates

Promising alternative methods for the modified biomolecule synthesis of a novel compound with the desired physicochemical properties are conjugation, polymerization, or grafting; such approaches are used for yielding improved properties, *e.g.*, sensorial and nutritional, with specific applications (Czubinski and Dwiecki 2016). Protein is present in eggs, meat, cereals, milk, legumes, and oilseed, with diversified amino acid sequences and compositions, making them different in terms of protein function, structure, and conformation (Ozidal *et al.* 2013). Protein is a major component, with different functions as well as allowing a different compound to be more effective in its role as an anti-oxidant and is widely applicable in the biomedical and pharmaceutical industries (Phillips and Williams 2011; Liu *et al.* 2015).

Table 2. Protein-Polyphenol Conjugation Changes and Methods in their Function

Conjugation Methods	Proteins	Polyphenols	Reaction Conditions	Influence on the Functional Properties/Bioactivities of the Conjugates	References
Enzymatic reactions	β -casein and β -lactoglobulin, cuttlefish skin gelatin, α -lactalbumin, lysozyme, and bovine serum albumin (BSA), gelatin	Caffeic acid, gallic acid, catechin, tannic acid, and ferulic acid, chlorogenic acid, catechin	Tyrosinase (pH 8.0) and laccase (pH 5.0), at 40 °C for 24 h, bubbled with oxygen, laccase, pH 5.0, at 40 °C for 1.5 h, bubbled with oxygen, polyphenol oxidase, pH 6.0, at 40 °C for 2.5 h, bubbled with oxygen, laccase, pH 7.0, at 20 °C for 24 h, under atmospheric air	Reduced solubility and in vitro digestibility, Improved gelling properties, reduced solubility, Increased inhibitory activity toward oxidation of low-density lipoprotein,	Chung <i>et al.</i> (2003); Prigent <i>et al.</i> (2003); Fan <i>et al.</i> (2018a, b); and Velickovic and Stanic-Vucinic (2018)
Alkaline reaction	Porcine plasma protein hydrolysates, β -lactoglobulin, lactoferrin, ovotransferrin, fish myofibrillar protein, cuttlefish skin gelatin, soy protein, lysozyme	Tannic acid and chlorogenic acid, caffeic acid, gallic acid, catechin, caffeic acid, ferulic acid, tannic acid, and catechin, tannic acid, chlorogenic, and caffeic acid, m-, o-, p- dihydroxy benzenes, ferulic, and gallic acid	pH 9.0, at 25 °C under atmospheric air for 24 h, pH 8.5, at 25 °C under atmospheric air for 24 h, pH 9.0, at 25 °C under atmospheric air for 24 h, pH 9.0, at 25 °C under atmospheric air for 24 h, pH 11.0, at 25 °C for 1 h with continuous oxygen purging, pH 9.0, at 40 °C, bubbled with oxygen for 1 h, pH 9.0, at room temperature with free exposure to air for 24 h, pH 9.0, at room temperature with free exposure to air for 24 h	Improved antioxidant activity, enhanced surface hydrophobicity, Improved antioxidant activity, and emulsifying property, Improved antioxidant and thermal stability, improved antioxidant activity, improved antioxidant activity, enhanced mechanical properties of protein film, improved antioxidant activity, and emulsifying property, reduced solubility and in vitro digestibility, increased surface hydrophobicity.	Rawel <i>et al.</i> (2001); Rawel <i>et al.</i> (2002); Prodpran <i>et al.</i> (2012), Aewsiri <i>et al.</i> (2013); You <i>et al.</i> (2014); Liu <i>et al.</i> (2016a); El-Maksoud <i>et al.</i> (2018); and Chen <i>et al.</i> (2019a, b)

Free radical grafting	Whey protein isolate, ovalbumin, egg white protein, Ovo transferrin	EGCG, catechin, epigallocatechin, and EGCG, catechin, catechin	H ₂ O ₂ /ascorbic acid, under atmospheric air at 25 °C for 24 h, H ₂ O ₂ /ascorbic acid, under atmospheric air at 25 °C for 24 h, H ₂ O ₂ /ascorbic acid, under atmospheric air at 25 °C for 24 h, H ₂ O ₂ /ascorbic acid, under atmospheric air at 25 °C for 24 h	Improved antioxidant activity, improved antioxidant activity, enhanced surface hydrophobicity, Improved antioxidant activity, improved antioxidant activity	You <i>et al.</i> (2014); Gu <i>et al.</i> (2017); and Feng <i>et al.</i> (2018)
Noncovalent complex formation	α-lactalbumin, lysozyme, and BSA, whey protein isolate and casein, egg white protein, BSA, milk β-lactoglobulin, α-lactalbumin, lysozyme, and BSA	Chlorogenic acid, chlorogenic acid, EGCG, ferulic acid, tea polyphenols, procyanidins	pH 9.0, at room temperature with free exposure to air for 24 h, pH 7.0, at 25 °C for 24 h, pH 7.0, at 25 °C for 24 h, pH 7.0, at 25 °C for 24 h, pH 7.0, at 25 °C for 24 h, pH 7.0, at 25 °C for 24 h	Reduced protein solubility, improved solubility, foaming properties, antioxidant activity, increased digestibility, improved antioxidant activity, enhanced surface hydrophobicity, improved thermal stability, improved thermal stability and antioxidant activity, reduced protein solubility, improved foam stability	Prigent <i>et al.</i> (2007); Prigent <i>et al.</i> (2009); Kanakis <i>et al.</i> (2011); Ojha <i>et al.</i> (2012); Yin <i>et al.</i> (2014); and Jiang <i>et al.</i> (2018)

Phenolic compounds are natural antioxidants with an aromatic ring attached to hydroxyl groups, which prevents or delays oxidative losses. Some of the attained properties *via* protein-polyphenol conjugation are antimicrobial, antioxidant, and bacterial enzyme inhibition as well as decreasing the oxidative damage level in living cells, DNA, protein, carbohydrates, and lipids (Maqsood *et al.* 2012; Liu *et al.* 2017). The protein-polyphenol interaction improves the quality of food, and a non-polar polyphenol-protein conjugate improves the surface hydrophobicity and protein emulsification. Protein-polyphenol conjugates are covalently formed *via* enzymatic (polyphenol oxidase, laccase, tyrosinase, *etc.*) or non-enzymatic (alkaline reaction or free-radical grafting) methods (Gu *et al.* 2017; Liu *et al.* 2017) (as shown in Table 2).

FACTORS AFFECTING THE FORMATION OF PROTEIN POLYPHENOL CONJUGATES

The two types of factors influencing protein-polyphenol conjugates include extrinsic factors (temperature and pH) and intrinsic factors (polyphenol/protein type and structure), which determine the covalent/noncovalent interactions (Ozidal *et al.* 2013; Czubinski and Dwiecki 2017). The two major effects that affect the protein-polyphenol interaction are temperature and pH. Temperature affects the protein-polyphenol interaction *via* hydrogen bonding or hydrophobic-hydrophobic interactions, as heat changes the protein conformation, exposing hydrophobic sites, which influences the hydrophobic compounds to bind (Kulmyrzaev *et al.* 2005). The hydrophobic effect between curcumin and casein has been observed, and the interaction of caffeic acid with tyrosine, lysine, and tryptophan residues was observed with ionic/hydrogen bonding at a temperature between 10 to 45 °C; as the temperature was increased to 40 to 90 °C, the binding tendency of bovine serum albumin (BSA) to polyphenols decreased (Bonomi *et al.* 1988; Suryaprakash *et al.* 2000; Rawel *et al.* 2005). The amount of hydrogen binding of neochlorogenic acid with sunflower seed protein rapidly decreased as the temperature increased from 30 to 55 °C, as heating at a particular temperature causes the exposure of binding sites *via* denaturation and conformational changes (Bourvellec and Renard 2012; Ozidal *et al.* 2013). Covalent interactions between proteins and polyphenols can be induced by heat, as polyphenol oxidation occurs, which results in quinone formation. In addition, at an alkaline pH, polyphenols oxidize to quinones, which covalently react with proteins, showing that the pH influences the protein and polyphenol structure and conformation (Rawel *et al.* 2005; Ozidal *et al.* 2014).

APPLICATIONS OF PROTEIN-POLYPHENOL CONJUGATES

Protein-based Films

Protein-based films are of great interest in terms of developing food packing materials, as they have renewable, biodegradable, and eco-friendly properties; however, they have lesser water vapor barrier properties, as they are a hydrophilic type of matrix (Prodpran *et al.* 2012; Nagarajan *et al.* 2015). Many hydrophobic substances, *e.g.*, oil and fats, are added to the film-forming solution, and their incorporation overcomes oxidation, producing discoloration and off-odor to overcome this problem (Prodpran *et al.* 2012). Myofibrillar and legume proteins are soluble in an alkaline environment, and polyphenol is added, so their oxidation occurs, and a protein-polyphenol conjugate is formed

(Kaewprachu *et al.* 2017). Plant extracts can be incorporated in protein films as an antioxidant (Adilah *et al.* 2018). The addition of a catechin-Bradon extract conjugate not only enhances the antioxidant properties but also the antimicrobial activity of fish myofibrillar film. In addition, soy protein isolate film and fish gelatin are enhanced when conjugated with mango kernel, as they show better antioxidant activity and tensile strength (Kaewprachu *et al.* 2017; Adilah *et al.* 2018). The conjugation of EGCG and fish skin gelatin was found to enhance its antioxidant properties and tensile strength and the water vapor barriers and gelatin film packing of chicken skin oil with EGCG decreased lipid oxidation in storage compared to polyethylene packing, so these packings are advised for oil and fat storage (Nilsuwan *et al.* 2019).

Emulsions

Protein-polyphenol conjugates, having enhanced emulsifying and antioxidative abilities, are used to improve food emulsion stability, *e.g.*, a catechin-ovalbumin conjugate enhances the emulsion stability fish oil, which was produced by free-radical grafting, and the modified emulsions had better storage stability, lower lipid oxidation, and smaller droplet size (Feng *et al.* 2018). The modified protein surface hydrophobicity improved the emulsifying activity and the conjugated droplets repulsion improved the emulsion stability, as a WPI-EGCG conjugate prevented fish oil deterioration and prevented fish oil lipolysis (Liu *et al.* 2016a,b; Fan *et al.* 2018a,b). The oxidized tannic acid-gelatin conjugate stabilized menhaden oil-in-water emulsion and showed less phase separation and lipid oxidation compared to unmodified gelatin and a fish oil emulsion had better oxidative stability with the presence of a B-lactoglobulin-green tea polyphenol conjugate, having the same droplet size (Aewsiri *et al.* 2009; Von Staszewski *et al.* 2011). The oil-water emulsion was physically stabilized by a rice hull phenolic extract-rice bran protein hydrolysate conjugate, which made a rapeseed oil emulsion less flocculated and coalescence with a smaller oil droplet size (Cheetangdee and Benjakul 2017; Li *et al.* 2019). Additionally, many covalent polyphenol-protein conjugates, *e.g.*, α -lactalbumin-catechin, lactoferrin-chlorogenic acid, β -lactoglobulin-catechin, catechin-egg albumin protein, and lactoferrin-EGCG, are used as antioxidants in an oil-in-water emulsion (Yin *et al.* 2014; Yi *et al.* 2015). Protein-polyphenol conjugates with enhanced interfacial properties can be absorbed on the oil surface and make the oil droplets stable from electrostatic repulsion and steric hindrance, as shown by milk proteins (Staszewski *et al.* 2012). Therefore, protein-polyphenol conjugates could be used as antioxidant emulsifiers in food products.

Protein Gels

Protein gel is formed by a three-dimensional protein chain network connected *via* covalent/noncovalent interaction and can be produced by different methods, *e.g.*, environmental changes, mineral addition, pH adjustments, and enzyme cross-linking (Liu *et al.* 2017; Quan and Benjakul 2019). Oxidized polyphenols are primarily used in protein cross-linkers, which enhance gel formation, *e.g.*, β -lactoglobulin-green tea polyphenol conjugate increased B-lactoglobulin gelatin by lowering the gelling time and temperature (Balange and Benjakul 2009a,b,c; Staszewski *et al.* 2011, 2012; Velickovic *et al.* 2017). The change in the gelling properties of yogurt could be because of the addition of cocoa polyphenol, which increases the syneresis and decreases the elasticity, and gelatin could be covalently modified with tannic acid, yielding an improved rigid structure (Yildirim-Elikoglu and Erdem 2017). Additionally, the gelatin hydrogel matrix could be improved by tannic acid/caffeic acid/*Fructus chebulae* extract by producing a compact structure. The

addition of oxidized polyphenols could increase the strength of bigeye snapper surimi gel and a similar increase could be seen with the addition of coconut husk extract to sardine surimi (Balange and Bejakul 2009a,b,c). Tea polyphenol modification to egg albumen proteins enhances gurnard surimi gels, and studies showed that protein-polyphenol conjugates have a binding ability with both hydrophilic and/or hydrophobic compounds (Chanphai *et al.* 2018; Chanpai *et al.* 2019; Zhao and Sun 2018). Moreover, protein-polyphenol conjugated emulsifiers for delivering encapsulated bioactive compounds stabilizes the β -carotene oil-in-water emulsion by improving their stability in GIT conditions.

Whey Protein-Polyphenol Conjugates and Complexes

Whey proteins (WP), a globular protein series, are found in milk, *e.g.*, α -lactalbumin, BSA, and β -lactoglobulin, which are used as a functional ingredient in the food industry, primarily as a foaming and emulsifying encapsulating agent or as a gel structure in different products (O'Connell and Fox 1999; Krolczyk and Janiszewska-Turak 2016). Whey proteins generate peptides, which have potentially beneficial properties, *e.g.*, antioxidant, anti-hypertension, and cholesterol lowering. This gives them health benefits, but their consumption is limited, as they are functionally deteriorated because of numerous physical influences, *e.g.*, temperature, pH, and ionic strength (Buggy *et al.* 2018; Minj and Anand 2020; Baba *et al.* 2021; Koningsveld *et al.* 2002; Tokmakov *et al.* 2021). These processes change the structure of WPs *via* biological activity alteration, so their health properties are changed, as there are numerous physicochemical modifications that need to be investigated to overcome these flaws and increase the functions of WPs (Madhan *et al.* 2005; Cao *et al.* 2018; Rasouli *et al.* 2019). The techno-functional properties of WPs are improved by their linking to food-grade products and are bonded to polysaccharides and polyphenols *via* covalent linkages or noncovalent linkages (Cao *et al.* 2018; Setiowati *et al.* 2020) and thereby improve the techno-functional qualities of the protein with the biological qualities (Doost *et al.* 2019; Khalifa *et al.* 2019; Pessato *et al.* 2019; Wang *et al.* 2020a,b; Zhong *et al.* 2021). Recently, there have been advancements in modified protein manufacturing that resulted in enhanced biological and techno-functional properties, with rapid advances in food science technology applications (Chen *et al.* 2021). This review presents and evaluates studies on the characterization, fabrication, and biological and techno-functional properties of WP-polyphenol complexes and conjugates, particularly their possible applications in food industries. The aims and reviews of this article are summarized in Fig. 1, which is also helpful for researchers in utilizing whey protein-polyphenol ingredients in food products and identifies the requirements for further research.

Whey Protein-Polyphenol Interactions

Whey proteins and polyphenols are linked *via* noncovalent (physical) or covalent (chemical) interactions and depend upon the nature and linkage type of the whey protein and polyphenols for determining the biological and functional properties of the complexes/conjugates produced. The noncovalent bonding is reversible and weak, which includes hydrophobic attraction, hydrogen bonding, van der Waals forces, and electrostatic links, and contains hydrophobic residues, *e.g.*, valine, isoleucine, leucine, and tryptophan, which form hydrophobic links with aromatic non-polar rings in the polyphenols (Yildirim-Elikoglu and Erdem, 2017; Charlton *et al.* 2002; Damodaran *et al.* 2007; Tang *et al.* 2016). Additionally, hydrogen bonds can be formed between the polar groups of WPs and the

polyphenol hydroxyl groups. When the polyphenols are charged, electrostatic interactions could be present on oppositely charged protein groups (Zhang *et al.* 2010).

Covalent bond conjugates are formed between WPs and one or more polyphenols, which are irreversible and strong and involve the nucleophilic groups of the WPs, *e.g.*, -SH or -NH₂ groups. Some common chemical reactions involved are alkaline and free radical-induction methods, but various other methods are also used. In each method, the reaction conditions should be monitored appropriately to obtain the desired structure, function, and composition of the conjugates; the term pure refers to commercially procured polyphenols with a purity level greater than 98%.

Table 3. Whey Protein-polyphenol Covalent Conjugation: Detection, Functional, and Production Properties

Protein-polyphenol Interaction	Analytical Techniques	Method of Production	Functional Properties	References
WPI-EGCG	SDS-PAGE; CD; FTIR (Maksoud <i>et al.</i> 2018); ESI-MS	Free radical	Improved antioxidant activity Improved inhibition of lipolysis	Fan <i>et al.</i> (2018a, b)
WPI-Proanthocyanidins	CD; FTIR; NMR; UVS; FS; SDS-PAGE	Free radical	Improved antioxidant activity; improved oxidative and thermally stabilized emulsions	Chen <i>et al.</i> (2021)
WPI-Chlorogenic acid	SDS-PAGE; CD; FTIR; DSC	Free radical	Improved digestibility, solubility, and emulsifying properties reduced allergenicity	Xu <i>et al.</i> (2019a,b,c)
β -LG-EGCG/Chlorogenic acid	FS; CD; FTIR; MALDI-TOF-MS; SDS-PAGE	Free radical	Improved thermal stability, antioxidant properties and reduced allergenicity	Wu <i>et al.</i> (2018a,b)
α -LA-catechin	CD; ESI-MS	Free radical	Improved antioxidant activity and stability of nano emulsions	Yi <i>et al.</i> (2016)

β -LG-catechin	SDS-PAGE; CD; ESI-MS	Free radical	Improved encapsulating and antioxidant properties	Yi <i>et al.</i> (2015)
β -LG-Caffeic Acid	MALDI-TOF MS, FS, CD; DSC	Alkaline	Improved water solubility/ improved thermal stability	EI-Maksoud <i>et al.</i> (2018)
Lactoferrin-chlorogenic acid/ Gallic acid	SDS-PAGE; MALDI-TOF-MS	Free radical	Improved emulsifying and antioxidant properties	Liu <i>et al.</i> (2015)
Lactoferrin-chlorogenic acid/ EGCG	FTIR; CD	Alkaline	Improved thermal stability	Liu <i>et al.</i> (2016a, b)
β -LG-EGCG	SDS-PAGE and MALDI-TOF-MS; FS	Alkaline	Improved antioxidant activity	Tao <i>et al.</i> (2019)
WP (α -LA; β -LG; lactoferrin)-EGCG	CD; DSC; MALDI-TOF-MS	Alkaline	Improved antioxidant activity and physical and chemical stability of emulsions	Wei <i>et al.</i> (2015)
Lactoferrin-chlorogenic acid/ EGCG	SDS-PAGE; CD; FS	Alkaline	Improved functional properties/ Stable β -carotene	Liu <i>et al.</i> (2016a, b)
WPI- Quercetin/ rosmarinic acid	UVS; ESI-Q-TOF; RP-HPLC	Alkaline	Improved antioxidant properties	Ali (2019)
WPI-Chlorogenic acid/ rosmarinic acid	UVS; RP-HPLC; UHPLC-ESI-TOF-MS	Alkaline	Improved antioxidant and antiviral properties	Ali and Elsharkawy (2018)
WPI- Rosmarinic acid	FS; HPLC	Alkaline/ enzymatic	Improved antioxidant and antimicrobial properties	Ali <i>et al.</i> (2018)
FS: fluorescence spectroscopy; UVS: ultraviolet-visible spectroscopy; FTIR: Fourier-transform infrared spectroscopy; CD: circular dichroism; MS: mass spectroscopy; MALDI-TOF-MS: matrix-assisted laser desorption/ionization-time of flight; ESI-Q-TOF: electrospray ionization quadrupole time of flight; HPLC: high-performance liquid chromatography; EGCG: epigallocatechin-3-gallate; and BSA: bovine serum albumin				

Table 4. Whey Protein-polyphenol Noncovalent Conjugation; Detection, Function, and Production Properties

Protein-polyphenol Interaction	Functional Properties	Analytical Technique	References
WPI-EGCG-Caffeic acid	Improved stability of polyphenols during <i>in vitro</i> digestion	FS	De Moraes <i>et al.</i> (2020)
WPI-caffeic acid/EGCG	Reduced allergenicity	Not performed	Pessato <i>et al.</i> (2019)
WPI - Chlorogenic acid	Improved solubility, foaming properties, Improved digestibility, Improved radical scavenging	FS, FTIR	Jiang <i>et al.</i> (2018)
β -LG/ α -LA/ BSA resveratrol	Improved antioxidant activity and stability	FS; CD; Docking	Cheng <i>et al.</i> (2018)
WP-carvacrol	Smaller particle size, better dispersion and film-forming capacity, Improved emulsifying property	Not performed	Wang <i>et al.</i> (2020a, b)
β -LG anthocyanins	Improved thermal, oxidative and photostability	FS; FTIR; CD; Docking	He <i>et al.</i> (2016)
β -LG anthraquinones	Improved hydro solubility	FS; FTIR; CD; Docking	Xu <i>et al.</i> (2019a, b, c)
α -LA-chalconoids	Improved functional and antioxidant properties	FS; FTIR; Docking	Jiang <i>et al.</i> (2020)
BSA-tea polyphenols	Improved encapsulating efficacy	UVS; FTIR; Docking	Chanphai and Tajmir-Riahi (2019)
β -LG-EGCG	Improved stability, improved sensory properties	FTIR; SDS PAGE	Shpigelman <i>et al.</i> (2012)
FS: fluorescence spectroscopy; UVS: ultraviolet-visible spectroscopy; FTIR: Fourier-transform infrared spectroscopy; CD: circular dichroism; MS: mass spectroscopy; MALDI-TOF-MS: matrix-assisted laser desorption/ionization-time of flight; ESI-Q-TOF: electrospray ionization quadrupole time of flight; HPLC: high-performance liquid chromatography; EGCG: epigallocatechin-3-gallate; and BSA: bovine serum albumin			

FOOD APPLICATION OF WHEY PROTEIN-POLYPHENOL (WP-P) CONJUGATES AND COMPLEXES

An important techno-functional property of proteins is water solubility. Determining their use in food products and the protein-polyphenol physical interaction alters the protein solubility, so their function is also changed. For example, the conjugation

of whey protein with chlorogenic acid increases the water solubility. This is probably because of the polyphenol binding to the protein surface, which increases the hydrophilicity and decreases the hydrophobic interaction of the protein molecules (Tsai and She 2006; Arimboor *et al.* 2011; Jiang *et al.* 2018). A visible decrease in the surface hydrophobicity of WP was observed after the formation of an EGCG and gallic acid complex, while the water solubility of β -lactoglobulin increased with the formation of resveratrol and anthraquinones complexes, primarily because of the surface hydrophobicity reduction after binding (Wang *et al.* 2014; Cao and Xiong 2017). The WP-P complexes also affect the protein interfacial properties, which influences their stability to form emulsions and foams, as there the isolate foaming capacity of WPs increased when a complex was formed with chlorogenic acid and EGCG (Jiang *et al.* 2018). Whey proteins have numerous charged groups, hydrogen receptors, and non-polar groups on their surface, which makes them available for hydrophobic interactions, electrostatic interactions, and hydrogen bonding (Bartolomé *et al.* 2000; Buitimea *et al.* 2016; Khalifa *et al.* 2018). Whey protein fibril complexes with carvacrol improves the film-forming ability, emulsifying ability, and their antimicrobial activity; WP complex formation with xanthone enhances the protein emulsifying property compared to WPs alone (Rahayu *et al.* 2020; Wang *et al.* 2020a,b). The emulsion stability was improved because of the reduction in hydrophobic attraction and increased electrostatic and steric repulsion between the protein-coated oil drops. Jiang *et al.* (2020) and Li *et al.* (2020) studied the increasing foam stability and capacity after complexation with proanthocyanidins in a dose-dependent way and these improvements were because of the increasing interfacial layer elasticity around the air bubbles or oil drops. The air bubbles inhibit them from coalescing or collapsing, and the increased emulsification is because of additional protein solubility after making the complex, as well as facilitating rapid protein transfer to the interfacial layers (Jiang *et al.* 2018). However, a protein-polyphenol complex must be carefully produced to obtain the desired interfacial properties (Zhan *et al.* 2020).

IMPACT OF WP-P ON HEALTH PROMOTING PROPERTIES

The whey protein-polyphenol complex has numerous effects on bioactive qualities; *e.g.*, the complex can reduce the antioxidant ability of the polyphenol because of the masking effect (Adrar *et al.* 2019). Additionally, a WP complex with EGCG enhances the antioxidant activity of the polyphenol during the long-term storage and GIT conditions. Furthermore, it was explained by Chen *et al.* (2019a,b) that this complex preserved up to 27% of the antioxidant ability for 7 d of storage. The radical scavenging ability of EGCG-WPI complexes could be increased *via* sonication after storage, while the pre-heating of the milk protein polyphenol mixture reduces its antioxidant ability (Relkin and Shukat 2012; Clion *et al.* 2019). This could be because of the thermal denaturation of the WPs, which increases polyphenol binding to the protein exposed hydrophobic patches; however, additional study is still required to understand the required techniques for the production of WP complexes (Papadopoulou *et al.* 2004; Bayraktar *et al.* 2019). The radical scavenging ability of polyphenols is further reduced with α -lactalbumin compared to lactoferrin and β -lactoglobulin, which could be because of the polyphenol binding tendency for WPs, while the antioxidant activity of some anthraquinones- β -lactoglobulin complexes was reduced because of the complex formation (Stojadinovic *et al.* 2013; Xu *et al.* 2019a,b,c). Some WP-P complexes showed improved antioxidant activity compared to

alone, *e.g.*, resveratrol, phenolic acids, and curcumin, and the antioxidant property and heat stability of resveratrol was increased in a β -lactoglobulin complex. This could be because of the proteins' ability to prevent the resveratrol from converting from the *trans* to *cis* form (Cheng *et al.* 2018; Guo and Jauregi 2018; Li *et al.* 2018; Wu *et al.* 2018a, b). The stability of grape skin anthocyanin extract was increased regarding color retention with different destabilizing agents, *i.e.*, by β -lactoglobulin and β -lactoglobulin-quercetin complexes, as it was protected by a polysaccharide, and is a method to release the protected quercetin from acidic stomach degradation. In addition, it has been indicated in multiple studies that the β -lactoglobulin polyphenol complex enhances the polyphenol availability (He *et al.* 2016; Mirpoor *et al.* 2017; Shafaei *et al.* 2017; Chanphai *et al.* 2018). Protein-polyphenol complexes decrease the allergenicity of WPs, *e.g.*, delphinidin-3-*O*-glucoside, theaflavins, and chlorogenic acid reduce β -lactoglobulins binding *via* IgE epitomes. The allergenicity of BSA and β -lactoglobulin was reduced by complexation with EGCG at different pHs and WP oral sensitization of WP was decreased when complexed with caffeic acid and EGCG (Pessato *et al.* 2018, 2019). As a result, many WPs are used as delivery systems to improve the function of polyphenols, and these systems are then used as functional food fortification for health benefits (Al-Hanish *et al.* 2016; Chanphai and Tajmir-Riahi 2019).

IMPACT OF WP-P ON TECHNO-FUNCTIONAL PROPERTIES

The covalent bonds of polyphenol-WPs modify their techno-functional properties; *e.g.*, WPs conjugation causes the partial unfolding of WP molecules and exposes the surface hydrophobic sites, which causes enhanced emulsifying, foaming, and solubility properties of the proteins (Temdee and Benjakul 2015; Xu *et al.* 2019a,b,c). Whey protein complexes with lotus seed proanthocyanidins enhance the emulsifying/oxidative/thermal stability of the WP, and the conjugation of WPs with mulberry polyphenols enhances the solubility and textural properties and decreases their ability to foam glycosylated end products (Khaifa *et al.* 2019; Chen *et al.* 2021). Different polyphenols, *e.g.*, gallic acid, chlorogenic acid, and EGCG, conjugate with lactoferrin *via* free radical scavenging, which enhances the emulsification and thermal stability as well as produces emulsions, and provides a resistance to salt, freezing, thawing, UV exposure, and thermal applications (Liu *et al.* 2015, 2017; Lang *et al.* 2019). Moreover, EGCG and chlorogenic acid prevent lactoferrin aggregation by producing steric/electrostatic repulsions in protein molecules and also change the protein isoelectric points to control pH-induced aggregation (Liu *et al.* 2015). β -Lactoglobulin-catechin conjugates improve the physicochemical stability of β -carotene, which results in storage carotenoid retention. In addition, α -lactalbumin-catechin conjugates also are stabilized chemically *via* the radical scavenging from surface polyphenols (Yi *et al.* 2014, 2016). β -Lactoglobulin-chlorogenic acid conjugates improve the stability and inhibit EGCG release, while BSA-caffeic acid conjugates have better antioxidant activity with enhanced chemical stability and resveratrol bioavailability (Liu *et al.* 2017; Fan *et al.* 2018a,b; Prasanna and Jing 2018). Whey proteins have improved interfacial stability with polyphenol conjugation, and when covalently linked covalently with EGCG, many WPs had enhanced interfacial properties compared to their noncovalent complexes (Wei *et al.* 2015). The improving tendency of WP-EGCG conjugates in terms of β -carotene emulsion stability is in the following order: β -lactoglobulin is greater or equal to α -lactalbumin, which is greater or equal to lactoferrin, as their functional properties depend on the polyphenol type (Ali 2019). In a study, a WP-rosmarinic acid conjugate,

made *via* enzymatic methods, had better antioxidant and antimicrobial activity than the conjugate made *via* an alkaline method, so it should be necessary to further study the protein, polyphenols, and enzymes used to form different conjugates. Whey protein-polyphenol conjugates have health benefits with enhanced biological activity, as these conjugates have increased antioxidant properties because of the surface hydroxyl groups after adding polyphenol (Fan *et al.* 2017). Whey protein-chlorogenic acid conjugates have increased antioxidant quality and digestibility but decreased allergenicity, and it was also found that β -lactoglobulin-caffeic acid conjugates were DPPH radical scavengers, with more caffeic acid molecules attached to proteins (El-Maksoud *et al.* 2018; Xu *et al.* 2019a,b,c). Conjugates produced *via* the free radical method, *e.g.*, β -lactoglobulin-chlorogenic acid/lactoferrin, have enhanced antioxidant properties; as such, EGCG- β -lactoglobulin conjugate is beneficial for LDL oxidation, inhibiting atherosclerosis, primarily because of the metal chelation and radical scavenging activity (Liu *et al.* 2015; Fan *et al.* 2017; Tao *et al.* 2019; Liang *et al.* 2007). Catechin- α -lactalbumin conjugate has an improved scavenging ability compared to its protein or polyphenol alone, which shows their synergistic effect, while a WP-onion extract/quercetin conjugate showed anticancer abilities in human lungs and WP-rosmarinic acid/ chlorogenic acid has improved antioxidant/antiviral activities compared to native proteins.

FUTURE DIRECTIONS

The biological and functional activities of protein-polyphenol complexes or conjugates depend on their nature and cross-linking method type, so more rational complexes or conjugates can be designed in the future. As such, a greater understanding is required in terms of the function structure relation of WP-P conjugates and complexes for specific applications, and modern techniques; *e.g.*, high-pressure processing and sonication, must be utilized for system performance. For example, sonication increased the thermal stability and solubility of a cyanidin-3-galactoside-soy protein conjugate, whereas the performance of an α -lactalbumin pelargonidin-3-glucoside conjugate was improved *via* the high-pressure method (Xu *et al.* 2018; Zou *et al.* 2019). Until now, there have been limited studies on the GIT biological activity and WP-P conjugate/complex health benefits, as their interaction may change the generated peptide, so in terms of altering the protein bioactivity and allergenicity, additional focus is required in this area.

CONCLUSIONS

1. Protein-phenolic interaction can be covalent or noncovalent, but most of the published studies have focused on the noncovalent interactions, mostly because of the lack of methods for covalent bond analysis. Some of the primary factors affecting protein-polyphenol reactions are the protein type and structure and various environmental factors, *e.g.*, pH, temperature, salt concentration, and presence of other reagents. As an exact process, the interactions are still unknown, and studies have been performed on the changes in the structural and functional protein properties.
2. Phenolic fortification could improve the health benefits of food products, but their interaction with food components (especially proteins) could have some undesirable

effects on protein digestibility and antioxidative activity. Therefore, additional studies and research are required regarding the protein-phenolic interactions under different environmental conditions for the development of phenolic functional foods with the desired nutritional benefits.

3. Proteins and polyphenols have specific functions, *e.g.*, proteins being able to produce gel or stabilize emulsion and polyphenols having bioactivity and antioxidant activity, which influences food quality. Protein-polyphenol interaction can occur *via* the enzymatic or non-enzymatic method which is affected by various environmental conditions such as pH, temperature, and structure of the protein/polyphenols.
4. Protein-polyphenol conjugates have improved emulsifying and antioxidative properties, which increases food efficacy, and must be further studied for developing products with enhanced functional, nutritional, and sensorial properties.
5. Whey protein-polyphenol produces conjugates or complexes *via* covalent/noncovalent bonding, which produces multiple food ingredients, *e.g.*, foaming agents, antioxidants, emulsifiers, and structure formers. Their performance depends on the production method and WP-P type and concentration. A detailed understanding of protein-polyphenol interactions is essential to producing novel ingredients in a food system application, as their internal food system behavior is still not known.
6. The effect on bioactivity and functional properties of the ingredients are barely understood during the preparation, storage consumption, and digestion.
7. As there are a variety of polyphenol structures and functions, a protein-polyphenol ingredient must be produced with wider functions and rational designing. However, it is also important that these methods are viable economically and meet the food requirements.

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