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Possibilities and limitations in the analysis of covalent interactions between phenolic compounds and proteins

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ABSTRACT

Besides all comprehensive studies on botanical, analytical, and nutritional aspects of polyphenols, knowledge on interactions of these bioactive compounds with other constituents in the plant, food, or even in the human body is still scarce. From the several possibilities of interactions, covalent reaction products seem to be the most important ones, as they irreversibly affect the properties of both reaction partners. The phenolic compounds are highly reactive and many of them are able to be oxidized to their corresponding semiquinones and quinones which are further capable of undergoing covalent reactions with an enormous number of nucleophiles. When taking a look at nature, prominent nucleophiles are selected protein side chains such as lysine or cysteine. In the case of a reaction between phenolic compounds and protein side chains, properties of the single proteins and correspondingly the whole organism (plant or animal) might be severely affected. Even the smallest amounts of interactions can be of great impact. However, analysis of such reaction products is complex and therefore challenging. This is due to the fact that phenolic compounds remain reactive after the addition to the protein. This minireview shortly describes the chemical background(s) of the reaction and illustrates the main challenges in the analysis of protein–phenol-adducts. Moreover, questions that have to be considered when analyzing protein–phenol reaction products are raised and an alternative analytical idea is presented.

1. Interactions between plant phenolic compounds and proteins

With regard to evolution, plant phenolic compounds are secondary plant metabolites providing many different functions in the plant such as coloring, UV-shielding, phytoalexins, and repellents to name but a few (Dixon & Paiva, 1995; Friend, 1979). When being consumed as plant-based food being a part of the human diet, they potentially promote an impact on the health aspects of the human body. Whereas a number of studies conducted over the last decade suggest health-beneficial effects, also some adverse implications (Crozier, Jaganath, & Clifford, 2009; Scalbert, Manach, Morand, Rémésy, & Jiménez, 2005) have been reported. However, in most studies chemical transformations during plant food preparation have not been considered carefully, and compounds are often regarded as pure isolates, even inert. Often neglected is the fact that they might interact with the other compounds, either already in the plant, during food processing, or even after consumption of plant-based food in the human body. Of the primary compound classes, proteins seem to be the interesting ones, as they can interact with phenolic compounds at different sites. A review of the literature reveals the following locations or categories for the interactions of phenolic compounds: 1. In the plant with storage and physiological active proteins present; 2. Interactions of plant phenolic compounds with food proteins during food processing, resulting in changes of food texture; 3. Interactions may further occur with food proteins or enzymes in the course of digestion in the gastrointestinal tract; 4. During digestion, proteins/enzymes secreted in the gastrointestinal tract may serve as interaction partners; 5. After absorption, plasma proteins can be targets, and finally 6. There are also interactions possible with proteins in target tissues/organs in the human body.

From a chemical point of view protein–phenol-interaction can occur in different ways: Similar to the typical protein–protein interactions, hydrogen and ionic bonding and hydrophobic and aromatic interactions are key elements. Besides the mentioned ones, covalent bonds might be of special interest, as such bonds are irreversible and influence the (chemical) properties of both reaction partners.

Due to their chemical structure, plant phenolic compounds are highly reactive as they can be easily oxidized enzymatically or non-enzymatically (Cilliers & Singleton, 1991; Friedman, 1996) (Fig. 1). In plant material, the resulting ortho- or para-quinones are primarily reacting with other quinones to produce dark melanin pigments (Friedman, 1997; Nicolas, Richard-Forget, Goupy, Amiot, & Aubert, 1994; Robards, Prenzler, Tucker, Swatsitang, & Glover, 1999). Also during thermal treatments (i.e. during food processing), formation of quinones is a key element and will lead to degradation products that may also serve as substrates for a following interaction (Buchner, Krumbein, Rohn, & Kroh, 2006). In the case of oxidizing monohydroxyl phenolic compounds such as ferulic or coumaric acid, the reaction

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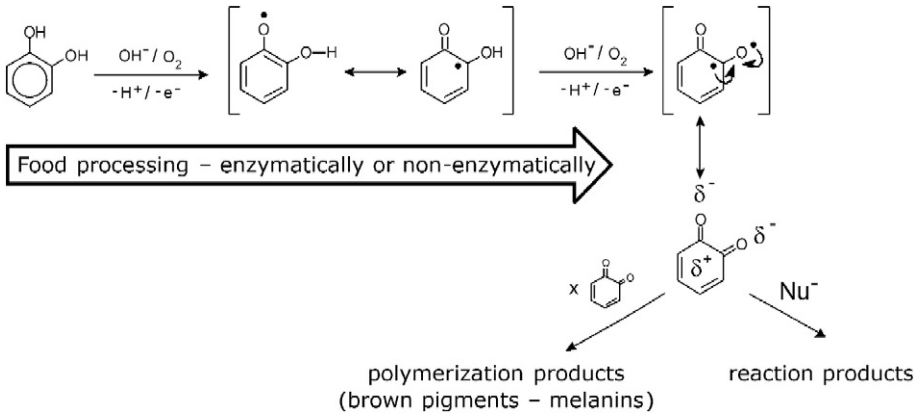

Fig. 1. Oxidation of a catechol moiety as a pre-requisite for the formation of reactions products.

proceeds via formation of semiquinones. The free radicals can then participate in non-enzymatic reactions, including polymerization, hydration, and disproportionation (Selinheimo, Autio, Kruus, & Buchert, 2007; Thurston, 1994). All the mentioned reactions are pre-requisites generating electrophilic species capable of underlying a nucleophilic (Michael-type) addition (Fig. 1). With regard to the structure of the phenolic compound (small phenolic acid, flavonoid, or complex procyanidin structure), it seems to be obvious that the more complex the phenolic compound, the more complex the reaction products will be. However, the oxidation of a catechol moiety has to be the primary step.

As mentioned above, proteins also provide nucleophilic protein side chains. Especially ε-amino groups, as present in lysine side chains, thiol groups, or the indole group of tryptophane, are the main examples. In one of the earliest investigations Mason and Peterson (1955) reported that the reaction of the quinone proceeds rapidly at neutral pH, as demonstrated for proline and nucleoprotamine. Besides, imidazole and disulfide groups have also been reported to be reaction partners (Kroll, Rawel, & Rohn, 2003; Loomis, 1974; Matheis & Whitaker, 1984a,b; Vithayathil & Murthy, 1972).

The consequences of such covalent reactions can be a change in protein structure with a corresponding change in the hydrophobic–hydrophilic properties of the protein derivatives, accompanied by a change in the solubility. These in turn may influence certain techno-functional properties (e.g. emulsion-, foam properties) (Gonzalez-Perez & Vereijken, 2007; Gonzalez-Perez et al., 2002; Prigent et al., 2003). Proteolytic digestion of such protein derivatives showed that at a lower degree of derivatization the tryptic degradation was adversely affected, whereas the peptic digestion declined with increasing modification. In vitro experiments illustrated that the digestion of derivatized lysozyme with enzymes of the gastrointestinal tract was adversely affected (Rawel, Kroll, & Rohn, 2001). However, due to differences in protein conformation, every protein might behave differently and a prediction of the degree of derivatization seems not possible.

Finally, the derivatization influences the biological activity of proteins/enzymes (Alberghina, 1964; Rohn, Rawel, & Kroll, 2001, 2002; Rohn, Rawel, Rober, & Kroll, 2005; Rohn, Rawel, Pietruschinski, & Kroll, 2001) resulting in a loss of nutritional quality of the proteins (Petzke, Schuppe, Rohn, Rawel, & Kroll, 2005; Rohn, Petzke, Rawel, & Kroll, 2006). In a rat growth and nitrogen balance study it was shown that a protein derivatization with chlorogenic acid affected the nutritional quality of beta-lactoglobulin (Petzke et al., 2005). Furthermore, properties of the phenolic compounds might be affected as well: e.g., the antioxidant activity of the phenolic compounds declined as a result of the covalent attachment to the protein (Rohn, Rawel, & Kroll, 2004; Rohn et al., 2005).

From all the above mentioned aspects it therefore seems mandatory knowing to which extent a protein is modified under certain conditions (e.g. food processing, consumption, digestion). Information about specific binding sites in the protein sequence to predict, correlate, or even model resulting effects on the protein conformation with the corresponding change of its properties would be very valuable. The locations where protein–phenol-interaction can occur (1. in the plant; 2. during food processing; 3. in the course of digestion in the gastrointestinal tract; 4. after absorption with blood plasma proteins; 5. proteins of target tissues in the human body) would greatly live from explanations about the properties of these neo-formed reaction products.

However, analysis of phenol–protein adducts is quite challenging, as the phenolic compounds remain reactive after the addition to the protein. Understanding the conditions required for the formation of covalent bonds or the biological consequences of the covalent modification of proteins by phenolic compounds is hindered by the lack of suitable analytical methods for evaluating the reaction products and the paucity of methods for purifying the products for a complete characterization (Hagerman, 2012). Strategies and methodologies are still not yet satisfying. In the next chapters, different approaches, as well as limitations, are described.

2. Analysis of protein–phenol-adducts

When investigating protein–phenol-adducts, there are several possibilities depending on the research strategy that is being applied. *Indirect* methodologies focus on the estimation of modified amino acids, analyzing the extent of phenolic compound bound, or evaluation of the resulting change in the properties of both compounds. *Direct* analysis is used to identify the specific binding site in a protein structure. Both approaches are quite challenging. Many limitations inhibit valuable results. Main reasons are the complexity of reaction products formed and the remaining reactivity of both reaction partners – proteins as well as phenolic compounds.

2.1. Indirect analysis

The easiest way to get an impression for the extent of a protein modification is the analysis of the amino acid side chains that remained unmodified after the reaction. This can be done either by full amino acid analysis and comparison with the results obtained for the unmodified protein (Petzke et al., 2005; Rohn et al., 2006), or by evaluating single amino acids with simple colorimetric or spectroscopic assays.

For example, there are numerous methodologies to determine free amino groups in a protein. One of the approaches appropriate for phenol–protein adducts is the derivatization with trinitrobenzenesulfonic acid (TNBS) in a 1% sodium dodecylsulfate (SDS) solution of the samples (Adler-Nissen, 1972). For modified whey proteins, it has been shown that the amount of free amino groups declines resulting from the modification with different plant phenolic compounds (Rawel, Kroll, & Hohl, 2001;