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Monitoring the apple polyphenol oxidase-modulated adduct formation of phenolic and amino compounds

Annika Reinkensmeier [a,b], Katrin Steinbrenner [a,c], Thomas Homann [a], Sara Bußler [d], Sascha Rohn [b], Hashadrai M. Rawel [a,*]

[a] Institute of Nutritional Science, University of Potsdam, Arthur-Scheunert-Allee 114-116, D-14558 Nuthetal, Germany
[b] Institute of Food Chemistry, Hamburg School of Food Science, University of Hamburg, Grindelallee 117, D-20146 Hamburg, Germany
[c] BioAnalyt GmbH, Rheinstr. 17, D-14513 Teltow, Germany
[d] Leibniz Institute for Agricultural Engineering Potsdam-Bornim e.V., Department of Horticultural Engineering, Max-Eyth-Allee 100, D-14469 Potsdam, Germany

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ABSTRACT

Minimally processed fruit products such as smoothies are increasingly coming into demand. However, they are often combined with dairy ingredients. In this combination, phenolic compounds, polyphenoloxidases, and amino compounds could interact. In this work, a model approach is presented where apple serves as a source for a high polyphenoloxidase activity for modulating the reactions. The polyphenoloxidase activity ranged from 128 to 333 nakt/mL in different apple varieties. From these, 'Braeburn' was found to provide the highest enzymatic activity. The formation and stability of resulting chromogenic conjugates was investigated. The results show that such adducts are not stable and possible degradation mechanisms leading to follow-up products formed are proposed. Finally, apple extracts were used to modify proteins and their functional properties characterized. There were retaining antioxidant properties inherent to phenolic compounds after adduct formation. Consequently, such interactions may also be utilized to improve the textural quality of food products.

1. Introduction

An increasing number of consumers has been observed to strive for a healthier lifestyle, being often reflected in their conscious and targeted measures to improve their dietary behavior. One of these trends, especially among the younger generation is the increasing demand for smoothies and related food products. The term smoothie describes a thick beverage made from fresh or frozen fruits (or vegetables) pureed in a blender. However, often these are mixed with dairy products such as milk, cream, or yoghurt. As these products are usually minimally processed, aspects with regard to product stability have to be considered. In this respect, attention has to be drawn to the enzymatically-induced transformation of secondary plant metabolites (SPM), associated with a high nutritional value and the specific sensorial properties of fruits/vegetables. In minimally processed products, enzymes are not inactivated and may be responsible for transformations of the constituents. Specifically, the secondary plant metabolite class of phenolic compounds is not the only determinant for the sensorial experience of the products, they are also found to have profound effects on human health due to their antioxidant, anti-inflammatory and antimicrobial effects (Kaume, Howard, & Devareddy, 2012; Liu, 2003; Schreckinger, Lotton, Lila, & de Mejia, 2010). They are preferential substrates for enzymatic transformations (Friedman, 1996).

As the counterpart to the phenolic compounds, the role of polyphenoloxidase (PPO) in (enzymatic) browning phenomena is very well documented and mainly contributes to a deterioration of plant product quality. Polyphenoloxidase, also known as tyrosinase, is a mixed-function oxidase possessing both monophenol monooxygenase activity (EC 1.14.18.1.) accompanied by an *o*-diphenoloxidase activity (EC 1.10.3.1./EC 1.10.3.2) (Mayer, 2006; Rawel & Rohn, 2010). These enzymes comprise a group of copper-containing proteins with binding sites for aromatic compounds and molecular oxygen, resulting in the insertion of oxygen

Abbreviations: DHAP, 2,5-dihydroxy acetophenone; L-DOPA, L-3,4-dihydroxyphenylalanine; DPO, diphenoloxidase; DMF, dimethylformamide; DMSO, dimethylsulfoxide; FA, ferulic acid; 3-(4-HP)PA, 3-(4-hydroxyphenyl)propionic acid; 4-MC, 4-methylcatechol; MCT, medium chain triglyceride; MBTH, 3-methyl-2-benzothiazolinone hydrazine; 4-MP, 4-methylphenol; PPO, polyphenoloxidase; SPM, secondary plant metabolites; L-TYR, L-tyrosine.

* Corresponding author.
E-mail address: rawel@uni-potsdam.de (H.M. Rawel).

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in a position *ortho* to an existing hydroxyl group in an aromatic ring, followed by the oxidation of the dihydroxybenzene structure to the corresponding semiquinones and quinones (Mayer, 2006). These (intermediate) oxidation products remain very redox-active and can further conjugate with other quinones to produce colored pigments (melanin's). Compartmentalization of enzymes and substrates and protection of substrates by derivatization have been discussed as the main mechanisms to control phenoloxidase activity (Sugumaran, Nellaiappan, & Valivittan, 2000).

However, the oxidation products are further capable of reacting with all kinds of nucleophiles. In this context, present research is aiming at the characterization of the interactions between PPO mediated polyphenol oxidation products with amino components (amino acids, peptides, and proteins), which are likely to occur under the prevailing conditions during the processing of fruits and vegetables in the presence of protein-rich ingredients (Ali, Homann, Khalil, Kruse, & Rawel, 2013; Rawel & Rohn, 2010). A few studies even describe the enzymatic oxidation of phenolic compounds as a new research field for protein-macromolecule conjugation (e.g., the role of ferulic acid in plant material cross-linking) that has attracted considerable attention (Figueroa-Espinoza, Morel, & Rouau, 1998; Figueroa-Espinoza & Rouau, 1999; Labat, Morel, & Rouau, 2001; Selinheimo, Lampila, Mattinen, & Buchert, 2008). The intention in those studies was to utilize the preliminary oxidation of the protein–polysaccharide bound phenolic structures (tyrosine, ferulic acid) by oxidative enzymes, which in turn results via further reactions in a cross-linking process. Specifically, a covalently bound oxidized phenolic compound may further react with an amino group or with a second oxidized phenolic compound of another macromolecule. This process promotes the cross-linking of the macromolecules present in the food system, thereby conferring semisolid textural attributes to e.g., dough or food gels. Among these oxidative enzymes, the use of laccase (EC 1.10.3.2) seems to be prevalent (Figueroa-Espinoza & Rouau, 1999; Figueroa-Espinoza et al., 1998; Labat et al., 2001; Selinheimo et al., 2008).

In the present study, apple was exemplarily chosen as a source for the well-known polyphenoloxidase activity and to characterize its behavior in modulating the reaction between amino components and selected phenolic compounds. Coupling of amino components (L-proline, 3-methyl-2-benzothiazolinone hydrazine) and phenolic oxidation products to produce chromogenic conjugates (Espin, Morales, Varon, Tudela, & Garciacanovas, 1995; Rzepecki & Waite, 1989) was used initially to determine the types of adducts formed in terms of their stability. The products were characterized by HPLC–MS. The main aim was to evaluate if PPO from apple can be used for modifying proteins. Finally, it was necessary to elucidate if this type of modification of milk proteins has any influence on the antioxidant properties of the phenolic compounds and if modified proteins can be used as innovative emulsifying agents providing protective effects against lipid oxidation.

2. Materials and methods

Commercially available apple samples of the cultivars 'Braeburn', 'Royal Gala', 'Golden Delicious', 'Granny Smith', 'Cripps Pink', and 'Jonagold' were purchased from local vendors (Potsdam, Germany). Mushroom tyrosinase was used as a reference enzyme (25 KU; Sigma Chemicals Co., St. Louis, MO, USA). A whey protein preparation (WP, Biopure, lot JE 002-8-415) was obtained from Davisco Foods International, Inc. (Le Sueur, MN, USA). This powder contained 98% protein (Kjeldahl analysis; Nx 6.38); 0.1% fat, 1.9% ash, and 4.7% moisture, as specified by the supplier. The major whey protein fraction was approx. 93.4% β-lactoglobulin as determined by SDS–PAGE. Commercially available medium-chain triacylglycerols (MCT, Linden Apotheke, Berlin, Germany) and food grade marigold concentrate (oleoresin, BioExtract, Bangalore, India) containing lutein esters (LE, ca. 40%, w/w) were used for the model emulsions. Lutein (20%) was provided by DSM Nutritional Products (Kaiseraugst, Switzerland). All other chemicals and HPLC solvents were reagent-grade or gradient grade, respectively.

2.1. Determination of the polyphenol oxidase (PPO) activities

2.1.1. Effect of storage temperature on PPO activity

The fresh 'Braeburn' apples kept at +4 °C, −20 °C and −80 °C before extraction, were allowed to thaw out (when necessary for 10–20 min), washed and cut into quarters. The core was removed, the apple quarters were pressed in fruit-juicing equipment (Phillips, HR7775, China) and the juice was collected in a container placed in an ice bath. Subsequently, the apple juice was filtered through a cloth sieve, diluted 1:15 with distilled water and immediately used for the PPO analysis (Section 2.1.4). The enzyme activity in 'Braeburn' apples kept at +4 °C, −20 °C and −80 °C was monitored over a period of three weeks.

2.1.2. Effect of centrifugal force on PPO activity

The fresh 'Braeburn' apples were kept at −20 °C before extraction. The juice was collected as described in Section 2.1.1 and aliquots of 2 ml were prepared. The retention of PPO activity in the 'Braeburn' juices/suspensions in comparison to untreated juice was investigated by applying different centrifugal forces (1000, 2000, 3000, 5000, 7000 and 10,000×*g*) on 2 mL of the juice for 5 min at 4 °C with the intention that even the finest particles are removed reliably. The PPO activity in diluted solutions was determined as described in Section 2.1.4.

2.1.3. Optimized sample preparation for the determination of the PPO activity

Apple samples of the different cultivars were kept at −20 °C before extraction. The juice was collected as described in Section 2.1.1, diluted with distilled water ('Braeburn' – 1:15, 'Royal Gala' – 1:12, 'Golden Delicious' – 1:8, 'Granny Smith' – 1:8, 'Cripps Pink' – 1:6, 'Jonagold' – 1:12) and the PPO activity was determined as described in Section 2.1.4. All further experiments utilizing apple juice were conducted with the cultivar 'Braeburn' prepared as described in this section. Apple slurries remaining after the pressing of the juice from the cultivar 'Braeburn' were used for the determination of the monophenoloxidase activity (Section 2.1.6).

2.1.4. Diphenoloxidase (catecholase) activity assay

Diphenoloxidase (catecholase, DPO) modulates the conversion of a 1,2-dihydroxyphenol to the corresponding *o*-quinone (Supplementary material, Fig. S1). The protocol for determining its activity was adapted according to Schilling, Sigolotto, Carle, and Schieber (2008). Briefly, a mixture consisting of 1.5 mL reaction buffer [0.5 mmol L^{-1} sodium dodecyl sulfate in 30% 0.1 mol L^{-1} citric acid and 70% 0.2 M disodium phosphate; pH 6.5], 0.2 mL 0.5 M L-proline in reaction buffer, and 0.1 mL of an enzyme-containing sample solution was prepared. The reaction was started by adding 0.2 mL of 25 mM 4-methylcatechol dissolved in the buffer. The formation of a pink proline–catechol adducts (Supplementary material, Fig. S1) was recorded photometrically at 525 nm (ε = 1550 L mol^{-1} cm^{-1}) every 3 s for 6 min at room temperature. PPO activity was calculated from the slope within the initial linear range of the absorbance–time curve. For blank correction, the slopes of a sample blank (0.1 mL water instead of enzyme-containing sample solution) and a reagent blank (0.2 mL reaction buffer instead of 25 mM 4-methylcatechol) were