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Antioxidant capacity of phenolic compounds on human cell lines as affected by grape-tyrosinase and *Botrytis*-laccase oxidation

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ABSTRACT

Phenolic components (PCs) are well-known for their positive impact on human health. In addition to their action as radical scavengers, they act as activators for the intrinsic cellular antioxidant system. Polyphenol oxidases (PPOs) such as tyrosinase and laccase catalyze the enzymatic oxidation of PCs and thus, can alter their scavenging and antioxidative capacity. In this study, oxidation by tryosinase was shown to increase the antioxidant capacity of many PCs, especially those that lack adjacent aromatic hydroxyl groups. In contrast, oxidation by laccase tended to decrease the antioxidant capacity of red wine and distinct PCs. This was clearly demonstrated for *p*-coumaric acid and resveratrol, which is associated with many health benefits. While oxidation by tyrosinase increased their antioxidant activity laccase treatment resulted in a decreased activity and also of that for red wines.

1. Introduction

A lot of attention in recent years has been devoted to free radicals and their influence on different constitutions of cells, thus, accelerating their aging and destruction. Human metabolism generates different reactive oxygen species (ROS), which create oxidative stress and cumulative oxidative damage in various biological macromolecules (Halliwell, 1991). It is well established that an array of different compounds contained in human diet can block the harmful action of ROS and oxygen-generated free radicals. Among them, phenolic compounds are well-known for their potential to scavenge free radicals and ROS, thus, exhibiting numerous health benefits. Their positive impact on human health as cardioprotectives, neuroprotectives, antifungals, antimicrobials, antidiabetics, anti-inflammatories and antitumorals is based mainly on their strong antioxidative properties which originate from their phenolic structure. The most important aspect regarding the scavenging activity of radicals is the number and position of the aromatic hydroxyl groups in the phenolic compound. Resonance effects in the aromatic system resulting from substituents also play a vital role in the efficiency and mechanisms of radical scavenging (Quideau, Deffieux, Douat-Casassus, & Pouysegu, 2011; Rice-Evans, Miller, & Paganga, 1996).

Several classes of phenolic compounds, such as flavonoids, hydroxylated *trans*-stilbenes, hydroxycinnamic acids and benzoic acids, are present in many foods and beverages such as wine (Waterhouse, 2002). Their concentrations and composition determine the color, astringency and bitterness of the wine (Hosu, Cristea, & Cimpoiu, 2014). Phenolic compounds are natural substrates for polyphenol oxidases (PPOs) such as tyrosinase and laccase. Both are metalloenzymes containing copper (Claus & Decker, 2006). Tyrosinase occurs naturally in grapes (Fronk et al., 2015). The vast majority of laccases, however, originate from a fungal infection with *Botrytis cinerea*, which releases laccases into the grape (Claus, Sabel, & König, 2014). Tyrosinase, which belongs to the type-3 copper protein family, can catalyze the hydroxylation of monophenols as well as the oxidation of *ortho*-diphenols to *ortho*-quinones (Rolff, Schottenheim, Decker, & Tuczek, 2011; Solem, Tuczek, & Decker, 2016). Laccase, comprising a multicopper-center, lacks the ability to oxidize monophenols. However, it is able to oxidize a broad range of diverse polyphenols and other compounds by a radical mechanism resulting in the for-

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mation of quinones. Quinones, as highly reactive substances, are prone to non-enzymatic self-polymerization, which leads to the formation of dark colored pigments (Mishra, Gautam, & Sharma, 2013). This is one of the most undesirable processes that occurs during winemaking (Claus, Sabel, et al., 2014).

In a previous study, we described the effect of fungal model enzymes, particularly tyrosinase from *Agaricus bisporus* and laccase from *Polyporus pinisitus*, on the antioxidant activity of phenolic components (PCs). We showed that both tyrosinase and laccase had a strong influence on the antioxidative activity of PCs which lack adjacent aromatic hydroxyl groups. Tyrosinase increased the scavenging activity and the antioxidative capacity in the cell model. Laccase displayed the opposite effect, with the exception of phlorizin, for which laccase increased the antioxidative capacity in the cell model (Riebel et al., 2015). In this study, we investigated the influence of tyrosinase directly isolated from Riesling grapes and laccase isolated from *Botrytis cinerea* strain P16-14 on the antioxidative effect. For this purpose, we have chosen five red wine varieties to get an impression of the reaction in real wine conditions. For further detailed analysis and to get an explanation for the action of the PPOs we have additionally chosen 20 PCs which are present in wine and are substrates for PPOs.

2. Materials and methods

2.1. Chemicals

All reagents were of analytical grade. Polydatin, phlorizin, phloretin, caffeic acid, ferulic acid, *p*-coumaric acid, sinapic acid, caftaric acid, 3,4-dihydroxybenzoic acid, 2,5-dihydroxybenzoic acid, 4-hydroxybenzoic acid, gallic acid, ethylgallate, vanillic acid, syringaldehyde, kaempferol, quercetin, kaempferol-3-glucoside, rutin, cyanidin, cyanidin-3-glucoside, DPPH·, DMSO, sodium citrate and tyrosinase from *Agaricus bisporus* were purchased from Sigma-Aldrich (Taufkirchen, Germany). Resveratrol, malate, tartrate and citric acid were supplied by Roth (Karlsruhe, Germany), and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) from AppliChem (Darmstadt, Germany). The chemiluminescence dye 8-amino-5-chloro-7-phenylpyridol[3,4-*d*]pyridazine-1,4-(2*H*,3*H*)dione sodium salt (L-012) was obtained from Wako Chemicals (Neuss, Germany), and DMNQ and PMA from Calbiochem/Merck KGaA (Darmstadt, Germany). Hanks' balanced salt solution was purchased from Gibco Life Technologies GmbH, Eggenstein, Germany. Laccase from *Polyporus pinisitus* was kindly donated by Novo Nordisk Biotech.

2.2. Wine and grapes

The red wines used in this study were Lemberger, Spätburgunder (Pinot Noir), St. Laurent, Merlot and Regent, all were from the vintage 2011 and obtained from commercial wineries in Germany (Claus, Tenzer, et al., 2014).

Riesling grapes (harvested on August 3, 2012) were obtained from a local vineyard in Harxheim, Rhine-Hessen, Germany (winery Fleischer, Mainz, Germany).

2.3. Determination of total phenolic content

The concentration of the total phenolic content in the wine samples was determined with the Folin-Ciocalteu reagent, using ferulic acid as a standard (Claus, Tenzer, et al., 2014).

2.4. Tyrosinase extraction and purification from grapes

Riesling grapes were washed, frozen at −20 °C and stored at −80 °C until use. The extraction protocol was adapted from Lago-Vanzela, Pavezzi, Martin, Gomes, and Da Silva (2011) with minor modifications. Frozen grape berries were ground in liquid nitrogen using a porcelain pestle and mortar. This finely ground powder was dissolved in one volume (w/v) of 0.2 M phosphate buffer (pH 6.5) containing 0.5 M NaCl, 10 mM ascorbic acid, 1 mM EDTA, 1% Triton X-100 and 2% PVP. After incubation for 30 min in 4 °C, the mixture was centrifuged for 15 min at 2800*g* and 4 °C. For purification, fast protein liquid chromatography (FPLC) was performed on a Bio-Rad Duo Flow system (Bio-Rad, Munich Germany), using a Sephacryl S-200 column (GE Healthcare, Munich, Germany) with a flow rate of 0.8 mL/min in 0.1 M phosphate buffer (pH 8.0). All fractions (3 mL) were collected and tested for PPO activity with a dot blot using dopamine (5 mM) as a substrate in 0.1 M sodium citrate buffer, pH 5.0. The fractions containing tyrosinase were pooled and concentrated using Amicon Ultra-15 centrifugal filter units (MWCO 10 kDa) (Merck-Millipore, Darmstadt, Germany).

Tyrosinase from *Agaricus bisporus* was purchased from Sigma-Aldrich (Taufkirchen, Germany). This tyrosinase was used for the oxygen consumption assay due to the high amount that was necessary.

2.5. Laccase isolation and purification from a Botrytis cinerea strain P16-14

Botrytis cinerea strain P16-14 with a high laccase activity was isolated from a Riesling grape must (winery Heymann-Löwenstein, Mosel, Germany) and preserved as pure culture on potato extract agar plates at 4 °C. The identification of the fungus was performed using the method of Hirschhäuser and Fröhlich (2007) based on PCR amplification of the laccase *lcc2* gene. In order to produce extracellular laccase, the strain was cultivated on a modified Czapek-Dox medium in 1.8 L Fernbach culture flasks (Schott, Germany) containing 300 mL medium at 20 °C. The medium contained (g/L): sucrose, 30.0; glucose, 10.0; $NaNO_3$, 2.0; Na_2HPO_4, 1.0; $MgSO_4 \times 7\ H_2O$, 0.5; KCl, 0.5; $FeSO_4 \times 7\ H_2O$, 0.01; asparagine, 1.0; and $CuSO_4$, 0.004 in 0.1 M phosphate buffer (KH_2PO_4, 12.0 g/L; Na_2HPO_4, 1.58 g/L, pH 5.8). A quantity of 1.0% (v/v) of sterile-filtrated yeast nitrogen base (Sigma, Munich, Germany) was added after autoclaving. After 10 days, the culture medium was centrifuged at 20,000*g* for 30 min to remove spores and mycelium. The medium was lyophilized (Lyovac GT2, Steris, USA) and the dried sample was reconstituted by redissolving in 1% of the original volume with tartrate-malate buffer (30 mM, pH 3.5). The solution was then incubated with 1.0% of a sodium-calcium bentonite (NaCalit®, Erbslöh, Germany) at 30 °C and shaken with 100 rpm for 2 h. Bentonite and bound proteins were removed by centrifugation at 7000*g* for 10 min. The resulting supernatant was dialyzed overnight against tartrate-malate buffer (30 mM, pH 3.5, MWCO of 14 kDa) to remove salts and other medium components. Finally, the yields of several preparations were combined and concentrated by ultrafiltration using Vivaspin centrifugal filtration units (MWCO 30) (Sartorius, Germany). This step also removed small proteins and peptides.

Laccase from *Polyporus pinisitus* was kindly donated by Novo Nordisk Biotech (Bagsværd, Denmark). This laccase was used for the oxygen consumption assay due to the high amount that was necessary.

2.6. Electrophoretic analysis

Discontinuous SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed as follows: laccase samples were mixed