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Polyphenoloxidase from Riesling and Dornfelder wine grapes (*Vitis vinifera*) is a tyrosinase ☆

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ABSTRACT

Polyphenoloxidases (PPO) of the type-3 copper protein family are considered to be catecholoxidases catalyzing the oxidation of *o*-diphenols to their corresponding quinones. PPO from Grenache grapes has recently been reported to display only diphenolase activity. In contrast, we have characterized PPOs from Dornfelder and Riesling grapes which display both monophenolase and diphenolase activity. Ultracentrifugation and size exclusion chromatography indicated that both PPOs occur as monomers with M_r of about 38 kDa. Non-reducing SDS–PAGE shows two bands of about 38 kDa exhibiting strong activity. Remarkably, three bands up to 60 kDa displayed only very weak PPO activity, supporting the hypothesis that the C-terminal domain covers the entrance to the active site. Molecular dynamic analysis indicated that the hydroxyl group of monophenolic substrates can bind to CuA after the flexible but sterically hindering Phe 259 swings away on a picosecond time scale.

1. Introduction

Polyphenol oxidases (PPOs) comprise different type-3 copper proteins as tyrosinase and laccase. Tyrosinase occurs in almost all organisms (Claus & Decker, 2006; Halaouli, Asther, Sigoillot, Hamdi, & Lomascolo, 2006; Marusek, Trobaugh, Flurkey, & Inlow, 2006; Mayer, 2006; van Gelder, Flurkey, & Wichers, 1997) and catalyzes two processes, the hydroxylation of monophenols in the *ortho*-position (monophenolase or cresolase activity) and the oxidation of diphenols to *o*-quinones (diphenolase or catechol oxidase activity) (Ramsden & Riley, 2014; Rolff, Schottenheim, Decker, & Tuczek, 2011; Sánchez-Ferrer, Rodríguez-López, García-Cánovas, & García-Carmona, 1995). These highly reactive molecules react further in a non-enzymatic way to form melanin (Rolff et al., 2011). The discrimination between the two catalytic reactions seems to depend on the presence of bivalent ions and of phenolic amino acids covering the access to CuA which inhibits the monophenolase activity (Decker & Rimke, 1998; Rolff et al., 2011). Unlike tyrosinase, laccases exhibit no monophenolase activity but oxidize a wide spectrum of different polyphenols and other compounds by a radical mechanism (Claus & Strong, 2010; Mayer & Staples, 2002). Beside PPOs, the oxygen carrier hemocyanins belong to the type-3 copper protein family (van Holde, Miller, & Decker, 2001) and can be converted to PPOs by limited proteolysis, lipoproteins and SDS (Coates, Kelly, & Nairn, 2011; Decker & Rimke, 1998; Decker, Ryan, & Jaenicke, 2001; Pless, Aguilar, Falcon, Lozano-Alvarez & Heimer de la Cotera, 2003). The proteins of this family share similar active sites, which bind a dioxygen molecule between two copper atoms in a side-on coordination as deduced from crystal structures (Cuff, Miller, & Hendrickson, 1998; Magnus et al., 1994; Masuda, Momoji, Hirata, & Mikami, 2014; Matoba, Kumagai, Yamamoto, Yoshitsu, & Sugiyama, 2006; Virador et al., 2010).

PPOs are involved in various biological functions, coloring of skin and hair, wound healing in invertebrates, sclerotisation of arthropods after molting and defence against pathogenic invaders by encapsulation (Cerenius & Soderhall, 2004). Among plants PPOs are additionally responsible for the browning spots which are of economic disadvantage (Martinez & Whitaker, 1995). Especially highly reactive quinones resulting from PPO reaction and a modified phenolic composition can lead to altered nutritional and

Abbreviations: PPO, polyphenoloxidase; Ty, tyrosinase; CO, catecholoxidase; LC–MS/MS, liquid chromatography tandem mass spectrometry; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

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organoleptic properties concerning color, flavor, sensoric and antioxidative capacity (Núnez-Delicado, Serrano-Megías, Pérez-López, López-Nicolás, 2005).

Most PPOs from plants are considered to be catecholoxidases and catalyze only the oxidation of *o*-diphenols (Marusek et al., 2006; Mayer, 2006; van Gelder et al., 1997). They often occur as isoforms being responsible for different biological functions (Dirks-Hofmeister, Inlow, & Moerschbacher, 2012; Mayer, 2006).

Most plant PPOs are monomers with a molecular mass of about 70 kDa and a very similar molecular architecture (Dirks-Hofmeister et al., 2012; Halaouli et al., 2006; Marusek et al., 2006; Mayer, 2006; van Gelder et al., 1997). After proteolysis of the two N-terminal signal sequences with a complete length of about 100 amino acids one obtains a latent PPO with about 60 kDa. Its N-terminal domain with about 38–40 kDa carries the active site followed by a short linker domain connecting the N-terminal domain with the 20 kDa C-terminal domain (Marusek et al., 2006). The C-terminal domain is thought to shield access of phenolic substrates to the active site (Marusek et al., 2006). Removing the C-terminal domain by proteolysis or detergents such as SDS opens the entrance to the active site which results in activation of the N-terminal domain.

Recently, a PPO, which exhibits only diphenolase activity, was purified from Grenache grapes (*Vitis vinifera var.* Grenache) and its crystal structure was presented (Virador et al., 2010). On the other hand, *in vitro* an *o*-hydroxylation activity was reported for red grape skins (*Vitis vinifera var.* Cabernet Sauvignon) converting *p*-coumaric acid to caffeic acid, although a monophenol- or a flavonoid 3′-monooxygenase was assumed to catalyze this reaction (EC 1.14.18.1 or EC 1.14.13.21) (Arnous & Meyer, 2009).

Here we analyze enriched PPOs from two different grape varieties and demonstrate that they exhibit diphenolase as well as monophenolase activity. The N-terminal domain with 38 kDa is more active compared to the full-length PPO including the C-terminal domain and PPOs seem to occur as monomers. Molecular dynamic simulation demonstrates that a conserved phenylalanine residue covering the entrance in the crystal structure will move away to give access to CuA for the hydroxyl group of bound monophenols thereby enabling monophenolase activity.

2. Material and methods

2.1. Grapes and protein extraction

Riesling and Dornfelder grapes (harvested October 10th, 2012) were obtained from a local vineyard (Fleischer, Mainz, Germany). Mature Riesling grapes were harvested in Mainz; Dornfelder grapes were harvested in Harxheim, Rhinehessen, Germany. Grapes were washed and frozen at −20 °C until use. The extraction protocol was adapted from Lago-Vanzela, Pavezzi, Martin, Gomes, and Da Silva (2011) with few modifications. Briefly, grapes were blended for 30 s with 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 20 min at 4 °C the grape solution was centrifuged for 30 min at 5000 rpm and 4 °C.

For Dornfelder grapes an additional PVP precipitation was applied. PVP (10% [w/v]) was added, stirred for one hour and removed by centrifugation for 10 min at 5.000 rpm and 4 °C. Remaining PVP was removed by filtration through a 0.22 µm polyethersulfone-filter (Rotilabo sterile filter, Roth, Karlsruhe, Germany) before subjecting the sample to chromatography.

2.2. Size exclusion chromatography

For an initial purification step 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 0.5 ml/min in 0.1 M phosphate buffer (pH 8.0). All fractions were collected and tested for PPO-activity with a dot blot using dopamine (5 mM) as substrate in 0.1 M citrate buffer pH 5.0. The fractions containing activity were pooled and concentrated using Amicon Ultra-15 centrifugal filter units (MWCO 10 kDa) (Merck-Millipore, Darmstadt, Germany). For molecular weight determination the sample was chromatographed on a Superose 12 column (HR10/30) (GE Healthcare, Munich, Germany) with a flow rate of 1 ml/min in 0.1 M phosphate buffer pH 8.0.

2.3. PAGE

2.3.1. Native PAGE

Discontinuous alkaline PAGE (7.5% at pH 8.8 with a 3% stacking gel at pH 6.8) was performed. The marker employed was Liquid Mix (Serva, Heidelberg, Germany). Gel electrophoresis was performed with 100 V at 4 °C and gels were stained with colloidal Coomassie brilliant blue R-250 according to Kang, Gho, Suh, and Kang (2002).

2.3.2. SDS–PAGE

For discontinuous SDS–PAGE 12.5% gels were used with a 3% stacking gel. The marker utilized was Precision Plus Protein Standard (Bio-Rad, Munich, Germany). Protein samples were mixed in equal amounts with SDS sample buffer [0.125 M Tris (pH 6.8)/20% (v/v): glycerin/10% (v/v):2-mercaptoethanol/4% (w/v) SDS-solution with a small amount of bromphenol blue]. Before being subjected to gel electrophoresis, the samples were denatured for 10 min at 95 °C.

For performing SDS–PAGE under non-reducing conditions a SDS sample buffer without β-mercaptoethanol was used and samples were not heated. Gel electrophoresis was performed with 100 V at room temperature. Gels were stained with colloidal Coomassie brilliant blue R-250.

2.4. In-gel activity assay

Activity of enriched PPO preparations was measured in native PAGE gels using the diphenols L-3,4-dihydroxyphenylalanine (L-Dopa), caffeic acid, dopamine and monophenols tyrosine, tyramine, *p*-coumaric acid (Sigma–Aldrich, Steinheim, Germany). For activity measurements MBTH (3-methyl-2-benzothiazolinoe hydrazone) was added to increase sensitivity (5 mM MBTH, 5 mM substrate in 0.1 M citrate buffer (pH 5.0). Mushroom tyrosinase (Sigma–Aldrich, Steinheim, Germany) was used as reference. For investigation of activity in SDS–PAGE native PPO samples were used without being denatured.

2.5. Enzyme activity and protein concentration

PPO activity of crude grape extract and enriched PPO fractions was measured spectrophotometrically applying the MBTH-assay at 505 nm at 25 °C with dopamine as substrate. MBTH and substrate concentrations were 2.5 mM in 0.1 M citrate buffer pH 5.0. 1 U corresponds to 1 µmol product formation per minute. The enzyme activity was calculated based on Lambert–Beer's law with a molar extinction coefficient of 29.000 M^{-1} cm^{-1} for the dopaquinone-MBTH-product.

The protein concentration of crude grape extract and enriched PPO fractions was determined according to the Bradford-assay (Bio-Rad Munich, Germany). Calibration curve was measured using bovine serum albumin (BSA) as standard in the concentration range of 6–120 mg/ml with a correlation coefficient higher than