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Purification and characterization of tyrosinase from walnut leaves (*Juglans regia*)

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ABSTRACT

Polyphenol oxidase (PPO) is a type-3 copper enzyme catalyzing the oxidation of phenolic compounds to their quinone derivates, which are further converted to melanin, a ubiquitous pigment in living organisms. In this study a plant originated tyrosinase was isolated from walnut leaves (*Juglans regia*) and biochemically characterized. It was possible to isolate and purify the enzyme by means of an aqueous two-phase extraction method followed by chromatographic purification and identification. Interestingly, the enzyme showed a rather high monophenolase activity considering that the main part of plant PPOs with some exceptions solely possess diphenolase activity. The average molecular mass of 39,047 Da ($Asp^{101} \rightarrow Arg^{445}$) was determined very accurately by high resolution mass spectrometry. This proteolytically activated tyrosinase species was identified as a polyphenol oxidase corresponding to the known *jrPPO1* sequence by peptide sequencing applying nanoUHPLC–ESI-MS/MS. The polypeptide backbone with sequence coverage of 96% was determined to start from Asp^{101} and not to exceed Arg^{445}.

Introduction

Polyphenol oxidases (PPO) are metalloenzymes containing a type-3 copper center occurring in many organisms including plants, fungi and bacteria (Mayer, 2006; Selinheimo et al., 2007). Representatives of this class are catechol oxidases, tyrosinases and laccases. Tyrosinases, a class of bifunctional PPOs, use molecular oxygen to catalyze the oxidation of various monophenols to *o*-diphenols (cresolase/monophenolase activity; EC 1.14.18.1), and the subsequent oxidation of *o*-diphenols to the corresponding *o*-quinones (catecholase/diphenolase activity; EC 1.10.3.1). Catechol oxidase catalyzes exclusively the oxidation of *o*-diphenols to *o*-quinones, lacking the hydroxylation reaction (Mayer, 2006; Mayer and Harel, 1979). Laccases (EC 1.10.3.2) can oxidize a wide range of compounds including aminophenols, monophenols, *o*- and *p*-diphenols by removing single electrons from the reducing group of the substrate and generate free radicals (Mayer and Harel, 1979; Sanchez-Amat and Solano, 1997).

Melanines generated by polymerisation of quinones are dying-compounds which are responsible for the damage-induced browning of many fruits and vegetables (Martinez and Whitaker, 1995; Yoruk and Marshall, 2003). While the physiological function of PPOs in many plants is still not clear, there is strong evidence that PPOs play a key role in parasite and pathogen resistance in some species. Protective effects of PPOs have generally been attributed to the generation of reactive quinones (Steffens et al., 1994; Van Gelder et al., 1997; Vaughn et al., 1988).

Type-3 copper proteins contain two copper ions, each coordinated by three histidine residues. During the catalytic reaction, the type-3 copper center of tyrosinase exists in three different states. The reduced *deoxy* state [Cu(I)–Cu(I)] binds molecular oxygen and results in the *oxy* state [Cu(II)–O_2^{2-}–Cu(II)]. In the *oxy* state, peroxide is bound in a μ–η^2:η^2 bridging mode (Kitajima et al., 1989). The *met* state [Cu(II)–Cu(II)] is assumed as the resting

Abbreviations: ACN, acetonitrile; CT, charge transfer; ESI-MS, Electrospray Ionisation Mass Spectrometry; FPLC, Fast Protein Liquid Chromatography; IEF, isoelectric focusing; *Jr*, *Juglans regia*; nanoESI-QTOF, nanoElectrospray ionisation quadrupole-time-of-flight mass spectrometry; LC–MS/MS, liquid chromatography–mass spectrometry/mass spectrometry; MS, mass spectrometry; nanoUHPLC–ESI-MS/MS, nanoUltra high performance liquid chromatography–electrospray tandem mass spectrometry; PEG-4000, polyethylene glycol 4000; PMSF, phenylmethylsulfonyl fluoride; PPO, polyphenol oxidase; SDS, sodium dodecyl sulfate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamid gel electrophoresis; UV/Vis spectroscopy, ultraviolet/visible spectroscopy.

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state of the copper site, where Cu(II) ions are bridged by a water molecule or hydroxyl ion (Solomon et al., 1996).

The functional importance of the PPO-activity in walnut hull was first described in 1991 (Piffaut and Metche, 1991). Walnut (*Juglans regia*) PPO was attributed to possess a putative pathogenic resistance as early as 1911 (Cook et al., 1911). Walnut leaves have a high content of various polyphenols, some of which might be important in pathogenic resistance (Colaric et al., 2005; Solar et al., 2006). PPO from walnut leaves has been poorly studied in the past (Escobar et al., 2008; Piffaut and Metche, 1991). Escobar et al. (2008) demonstrated that PPO of walnut is encoded by a single gene, *jrPPO1*, which is constitutively expressed in all green, herbaceous tissues of walnut.

In this work a tyrosinase from walnut leaves (*J. regia*) is extracted and purified by means of fast protein liquid chromatography (FPLC) and characterized by molecular mass determination (SDS–PAGE, nanoESI-QTOF), isoelectrical focusing (IEF), UV/Vis spectroscopy and sequence analysis (nanoUHPLC–ESI-MS/MS). The purification method described in this paper allows a very efficient isolation of two forms from walnut tyrosinase, identified and characterized by mass spectrometry based methodology providing sequence information and the highly accurate mass.

Results and discussion

Extraction and purification of tyrosinase from J. regia

The applied extraction method was based on a technique described for a latent tyrosinase from mushrooms (*Agaricus bisporus*) and had proven to be effective (Mauracher et al., 2014). Several consecutive aqueous two-phase separations using triton X-114 and PEG-4000 (polyethylene glycol) resulted in a quantitative removal of hydrophobic dyes, non-target proteins and other hydrophobic compounds. Hence, the obtained polyphenol free and clear protein solution was very well suitable for the subsequent chromatographic purification steps.

Using a cation exchange column (SP-Sepharose) as a first purification step proved being very effective in terms of removing a major part of non-target protein. The target PPO eluted late in the sodium chloride gradient together with one of in total three co-eluted heme-proteins. This is clearly shown in Fig. 1A by following the absorption at 410 nm characteristic for prosthetic heme groups. Implying some characterization experiments (data not shown) it can be assumed that the interfering protein is a peroxidase. This was also observed by Trémolières and Bieth (1984), Rompel et al. (1999a, 2012). Fractions showing highest tyrosinase activity were pooled and loaded onto the second cation exchange column (MonoS) where two major forms of the tyrosinase, which are named after the chromatographic elution order, forms 1 and 2, were eluted consecutively but efficiently separated early in the gradient (see Fig. 1B). On the cation exchange column (MonoS) the remaining heme protein (peroxidase) could be very effectively separated from the tyrosinase (see Fig. 1B). For polishing reasons fractions of the two tyrosinase forms were separately applied to the same cation exchange column (MonoS) resulting in a very high purity of the two tyrosinase species (see Figs. 1C/D and 2).

Often isoforms of polyphenol oxidase were found during isolation and purification. Currently six amino acid sequences (PPO1 to

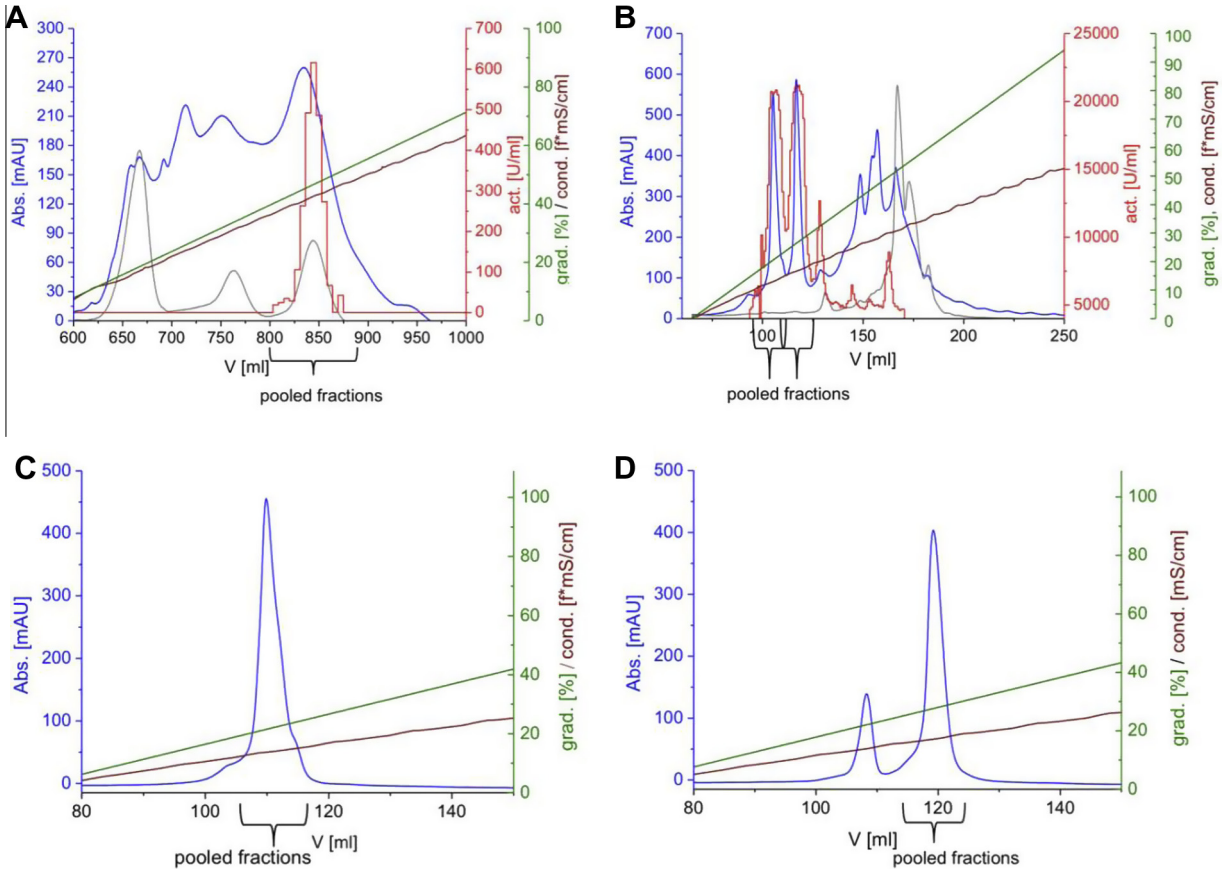

Fig. 1. Chromatographic runs (FPLC). (A) Cation exchange chromatography on SP-Sepharose. (B) Cation exchange chromatography on MonoS. (C) Cation exchange chromatography on MonoS *jrPPO1*(Asp^{101} → Pro^{444}). (D) Cation exchange chromatography on MonoS *jrPPO1*(Asp^{101} → Arg^{445}). Legend: ▬, UV absorbance at 280 nm [mAU]; ▬, UV absorbance at 410 nm [mAU]; ▬, monophenolase activity [U/ml]; ▬, gradient [% buffer B]; ▬, conductivity [mS/cm] (f = ~1.5).