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Single-step purification of peroxidase by 4-aminobenzohydrazide from Turkish blackradish and Turnip roots

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ABSTRACT

Peroxidases (PODs) were purified from the Turkish blackradish (*Raphanus sativus* L.) (TBR) and Turnip (*Brassica rapa* L.) using a simple and effective single-step method. An affinity resin was synthesised by coupling the 4-aminobenzohydrazide ligand and the L-tyrosine spacer-arm to CNBr-activated-Sepharose-4B. The purification factors for the TBR-POD and the Turnip-POD were 40.3-fold (with a yield of 10.6%) and 269.3-fold (with a yield of 9%), respectively. The molecular masses of the TBR-POD and Turnip-POD were approximately 67.3 and 65.8 kDa, respectively. For guaiacol, the K_m and V_{max} values were calculated as 24.88 mM and 3.23 EU/mL, respectively for TBR-POD and as 4.09 mM and 0.797 EU/mL for the Turnip-POD. For H_2O_2, the K_m and V_{max} values were calculated as 3.247 mM and 0.799 EU/mL, respectively for TBR-POD, and as 12.49 mM and 4.055 EU/mL, respectively for the Turnip-POD. Furthermore, 4-aminobenzohydrazide was determined to be a non-competitive inhibitor of TBR-POD and Turnip-POD.

1. Introduction

Plant peroxidases (PODs) (EC 1.11.1.7) catalyse the hydrogen peroxide-dependent oxidation of a wide variety of substrates, including phenol compounds (Marquez, Waliszewski, Oliart, & Pardio, 2008). PODs are found in all cells and play a role in many critical biological processes, such as the host-defence mechanism (Deepa & Arumughan, 2002). PODs are specific for a wide range of substrates and can be used in medicine for clinical tests. In the presence of an electron donating substrate, these enzymes catalyse the reduction of organic hydroperoxidases (Zilbeyaz, Kilic, Sisecioglu, Özdemir, & Alayli-Gungor, 2012). PODs are also used in organic synthesis for the production of various polymers, drugs and chemicals. Horseradish peroxidase (HRP), choloroperoxidase and TRP-POD have all been successfully used for the preparation of enantiomerically pure alkyl hydroperoxides and alcohols (Adam, Hoch, Lazarus, Saha-Müller, & Schreier, 1995). Recently, we reported the preparation of enantiomerically pure substituted phenylethyl hydroperoxides by kinetic resolution of racemic organic hydroperoxides with TRP-POD (Zilbeyaz et al., 2012).

Plant PODs have been purified from horseradish (*Armoracia rusticana*) roots (Lavery et al., 2010), from tree legume (*Leucaena leucocephala*) (Pandey & Dwivedi, 2011), from black gram (*Vigna Mungo*) husk (Ajila & Prasada Rao, 2009), from the caribbean plant (*Euphorbia cotinifolia*) (Kumar, Singh, Singh, & Jagannadham, 2011), from the leaves of the date palm (*Phoenix dactylifera* L.) (Al-Senaidy & Ismael, 2011), and from TRP-POD (Sisecioglu et al., 2010) using a variety of chromatographic techniques, such as ammonium sulphate precipitation, ion exchange column chromatography, Sephadex G100–200 gel filtration column and DEAE-Sephacel column chromatography. These studies have demonstrated that the plant peroxidase purification is an expensive and time-consuming procedure when traditional methods are used.

Affinity chromatography is a separation method that depends on molecular conformation. A chromatography resin is often used as the matrix for each protein. The resin surfaces depend on the specific ligands used for the purification of the desired protein. Such ligands connect to the proteins specifically and reversibly, similar to an antibody–antigen interaction. Aromatic compounds with an amino group can couple with agarose that has been activated with cyanogen bromide at an alkaline pH (Atasever, Ozdemir, Gulcin, & Kufrevioglu, 2013; Porath, 1968). A successful purification method for milk POD was reported by coupling the aromatic amines of the Sepharose 4B resin in a single step (Atasever et al., 2013).

Specifically, 4-aminobenzohydrazide is a well-known POD inhibitor that inhibits the myeloperoxidase enzyme; therefore, 4-aminobenzohydrazide and 4-aminobenzohydrazide derivatives can be used as the ligands for plant POD purification (Kettle, Gedye, & Winterbourn, 1997). TBR and Turnips are of the *brassicaceae* family and are cultivated in all regions of Turkey. Notably, this species is popular in Europe, particularly in the colder regions.

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The aim of this study was to develop an affinity method using aromatic amines for the purification of plant PODs. The TBR-POD and Turnip-POD were first purified in a single step using 4-aminobenzohydrazide and affinity chromatography. The 4-aminobenzohydrazide was determined to be a non-competitive and reversible inhibitor of the PODs. The purification method presented in this study is effective for the purification of other plant PODs and is less-time consuming than other methods.

2. Materials and methods

2.1. Enzyme source

Fresh TBRs and Turnips were obtained from a local market in Erzurum, Turkey. They were washed, drained, packed in polyethylene bags and stored at −20 °C until use.

2.2. Chemicals

CNBr-activated-Sepharose 4B, L-tyrosine, glycerol, 4-aminobenzohydrazide, guaiacol, hydrogen peroxide, potassium phosphate, and all other reagents used in the electrophoresis and protein assay were purchased from Sigma–Aldrich Co. (Sigma–Aldrich Chemie GmbH. Export Department. Eschenstrasse 5, 82024 Taufkirchen, Germany). All other chemicals were of analytical grade and were obtained from Merck and Sigma–Aldrich Co.

2.3. Preparation of Sepharose 4B-L-tyrosine-4-aminobenzohydrazide

The affinity resin was synthesised using 4-aminobenzohydrazide and L-tyrosine as the spacer arms for CNBr-activated-Sepharose 4B, according to a previously published procedure with a slight modification. The affinity gel was generated from the diazotisation of the 4-aminobenzohydrazide (Arslan, Nalbantoglu, Demir, Ozdemir, & Kufrevioglu, 1996; Atasever et al., 2013; Cuatrecasa, 1970).

2.4. Preparation of the crude extract

The crude homogenate was prepared according to the methods described previously by Sisecioglu et al. (2010). All homogenisation, centrifugation, dialysis and chromatography procedures were performed at 4 °C.

2.5. Peroxidase purification

The supernatant was applied to the Sepharose-4B-L-tyrosine-4-aminobenzohydrazide affinity column (1.3 × 10 cm) that had been equilibrated using a 10 mM phosphate buffer (pH 6.8). The affinity gel was washed with 400 mL of a 25 mM phosphate buffer (pH 6.8). The POD was eluted in a solution of 1 M NaCl/25 mM KH_2PO_4 (pH 6.8). The enzyme solution was then dialysed against a 10 mM KH_2PO_4 buffer (pH 6.8) for 2 h. The purity of the resulting fractions was assessed with sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE), which was performed as described by Laemmli (1975). The gels were stained using the silver staining method of Chevallet, Luche, and Rabilloud (2006). During the purification procedure, the protein concentration was determined using the Bradford (1976).

2.6. Peroxidase assay

The POD activities were determined using the measured absorbance change at 470 nm after the catalysis of guaiacol oxidation (Sisecioglu et al., 2010). The final reaction mixture contained 1 mL of 45 mM guaiacol, 1 mL of 22.5 mM H_2O_2 and 0.1 mL of the enzyme. The mixture was adjusted to 3 mL with the addition of 0.1 M phosphate buffer (pH 6.0). The absorbance was measured at 470 nm every 60 s. One unit of enzyme was defined as the amount of enzyme able to catalyse the oxidation of 1 μmol of guaiacol/min^{-1} at 25 °C (molar absorption coefficient, 5000 M^{-1} cm^{-1}).

2.7. Optimum pH

The optimum pH for the PODs of TBR and Turnip was determined in 0.1 M KH_2PO_4 buffer (pH: 5.0–8.0). The pH activity profiles for the crude extract PODs were generated with guaiacol (45 mM) and H_2O_2 (22.5 mM).

2.8. Optimum ionic strength

The ionic strength value for the PODs was determined using different concentrations of KH_2PO_4 buffers (25–150 mM) at the predetermined optimal pH.

2.9. Optimum temperature

The POD activity was determined at different temperatures using the guaiacol/H_2O_2 substrate. The enzyme activity was measured in the range of 0–80 °C.

2.10. pH stability

At a stable pH value, the POD activity was measured every 12 h for a period of 84 h in 4 °C. The process was carried out using a 10 mM KH_2PO_4 buffer, with a pH range between 5.0 and 8.0, separated by intervals of 0.5.

2.11. K_m and V_{max} values

The K_m and V_{max} values of the TBR-POD and Turnip-POD were estimated, using the guaiacol and H_2O_2 as substrates, from the Lineweaver–Burk plots. The reactions took place at 25 °C. The enzyme activity was measured at 470 nm for five different concentrations (9, 10.5, 12, 13.5 and 15 mM) of guaiacol while the H_2O_2 concentration was constant, and for five different concentrations (0.75, 2.25, 3.75, 5.25 and 6.75 mM) of H_2O_2 while guaiacol concentration was constant

2.12. Inhibition effects of 4-aminobenzohydrazide

The inhibition effects of the 4-aminobenzohydrazide on the purified POD were determined. To obtain the IC_{50} values, the POD activity was measured in the presence of different concentrations of 4-aminobenzohydrazide (0.28–1.42 mM) for the TBR-POD and of 4-aminobenzohydrazide (0.56–1.67 mM) for the Turnip-POD. A control sample without 4-aminobenzohydrazide was used to determine 100% activity, and an activity-[4-aminobenzohydrazide] plot was then drawn. For the determination of K_i, three different 4-aminobenzohydrazide concentrations were used: 0.28, 0.57 and 0.85 mM for the TBR-POD, and 0.84, 1.11 and 1.39 mM for the Turnip-POD. Guaiacol was also used as the substrate at five different concentrations (0.11–0.33 mM) for both the TBR-POD and Turnip-POD. Lineweaver–Burk plots (1/V-1/[S]) were obtained for the 4-aminobenzohydrazide; the K_i and the inhibition type were subsequently calculated from these plots. The data obtained was analysed by *t*-test, and the results are given as X ± SD.