Acta Biochimica Polonica

Vol. 55 No. 2/2008, 325–328

on-line at: www.actabp.pl

Regular paper

Polyphenol oxidase from wheat bran is a serpin

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Received: 15 November, 2007; revised: 27 March, 2008; accepted: 30 April, 2008
avaiable on-line: 26 May, 2008

Polyphenol oxidase (PPO; EC 1.10.3.2) was isolated from wheat bran by a procedure that included ammonium sulfate fractionation, batch adsorption by DEAE-cellulofine, CM-cellulofine column chromatography, DEAE-cellulofine column chromatography, preparative isoelectric focusing, adsorption on the membrane of a Vivapure Q Maxi H spin column, and heat treatment. These procedures led to 150-fold purification with 4.2% recovery. The PPO was homogeneous by SDS/PAGE. The relative molecular weight of the PPO was estimated to be 37 000 based on its mobility in SDS/PAGE. The isoelectric point of the PPO was 4.4. The K_m values of the PPO for caffeic acid, chlorogenic acid, pyrocatechol, 4-methyl catechol and L-DOPA as substrates were 0.077, 0.198, 1.176, 1.667 and 4.545 mM. The PPO was strongly inhibited by tropolone. The K_i value for tropolone is 2.2×10^{-7} M. The sequence of the 15 N-terminal amino-acid residues was determined to be ATDVRLSIAHQTRFA, which was identical to those of serpin from *Triticum aestivum* and protein Z from *Hordeum vulgare*. The PPO strongly inhibited the activity of trypsin, which is an enzyme of serine proteases; 50% inhibition was observed with 1.5×10^{-7} M PPO. The K_i value for PPO is 2.3×10^{-8} M. The wheat bran PPO should be a very important protein for protecting wheat against disease, virus, insect and herbivore damages by both the activities of PPO and protease inhibitor.

Keywords: polyphenol oxidase, serpin, wheat bran

INTRODUCTION

Polyphenol oxidase is an enzyme that catalyzes the hydroxylation of monophenols to *o*-diphenols and their oxidation to *o*-diquinones. While this enzyme is widely distributed in higher plants, its *in vivo* significance is not yet clear, although many possible roles have been proposed (Steffens *et al.*, 1994; Hind *et al.*, 1995; Trebst & Depka, 1995). Some authors have suggested that PPO plays a role in plant resistance against diseases (Ray & Hammerschmidt, 1998; Mazzafera & Robinson, 2000; Shi *et al.*, 2002; Witisuwannakul *et al.*, 2002; Melo *et al.*, 2006) and against insect herbivory (Felton *et al.*, 1989; 1992). Li & Steffens (2002) obtained direct evidence of such a role for PPO in plants. They observed that transgenic plants of tomato overexpressing PPO had a higher oxidizing capacity and showed increased resistance to *Pseudomonas syringae* pv. tomato. However, no previous study has identified a biological function for plant resistance against insect and herbivore damage.

On the other hand, we found that PPO of wheat bran inhibited trypsin activity. This fact may show that PPO defends plants from insect and herbivore damage.

We describe here the purification and properties of PPO from wheat bran.

EXPERIMENTAL PROCEDURES

Plant material. Wheat bran was obtained from Nisshin Flour Milling Co. (Sakaide, Japan).

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Abbreviation: DOPA, 3,4-dihydroxyphenylalanine; MCA, 4-methylcoumaryl-7-amide; M_r, relative molecular weight; NaOAc, sodium acetate; PAGE, polyacrylamide gel electrophoresis; PPO, polyphenol oxidase; SDS, sodium dodecyl sulfate.

Assay of PPO activity. The activity of PPO was determined as follows. The reaction mixture containing 5 mM of L-DOPA and enzyme solution in 2 ml of 50 mM NaOAc buffer, pH 6.0, was incubated at 30°C for 30 min. After incubation, the activity was determined by measuring the absorbance of the reaction mixture at 475 nm (A_{475}). One unit of PPO activity was defined as the amount of enzyme that provided an increase of 1 A_{475}/min under the conditions described above.

Determination of protein. Protein was determined by the method of Warburg and Christian (1942). The protein profiles in column chromatography were determined by measuring the absorbance of the eluates at 280 nm.

Electrophoresis. SDS/PAGE was performed on 7.5% gels according to the method of Laemmli (1970). After the run, the gels were stained for proteins with Phast Blue R (R-350).

The photograph of gel was taken by Vari Quest 26 (Fotodyne Incorporated, Hartland, WI, USA). The M_r of the purified enzyme was estimated according to the method of Laemmli (1970).

Estimation of the isoelectric point. The isoelectric point of the enzyme was estimated as described in the literature (Awdeh *et al.*, 1968; Fawcett, 1968). After electrofocusing for 24 h at 80 V, the gel was cut into 3-mm sections and each section was crushed in 2 ml of deionized water. The fractions were measured with regard to their enzyme activity and pH.

N-terminal amino-acid sequence analysis. The N-terminal amino acid sequence analysis was performed in an HP G1005A protein sequencing system (Hewlett-Packard Co., Palo Alto, CA, USA).

Assay of trypsin activity. The activity of trypsin (porcine pancreas trypsin) was determined as follows. The reaction mixture containing 0.1 mM of 4-methylcoumaryl-7-amide substrate (Boc-Gln-Ala-Arg-MCA, MCA substrate) and enzyme solution in 2 ml of 75 mM Tris/HCl buffer, pH 8.0, was incubated at 30° for 10 min. After incubation, the activity was determined by measuring the absorbance of the reaction mixture at 370 nm (A_{370}).

RESULTS AND DISCUSSION

Purification of PPO

Wheat bran was suspended in 20 mM phosphate buffer, pH 7.0. The supernatant was brought to 0.9 satn. with $(NH_4)_2SO_4$. After dissolving the precipitate in 20 mM phosphate buffer, pH 7.0, the enzyme solution was dialyzed against 20 mM Tris/HCl buffer, pH 8.0, and DEAE-cellulofine resin was added to the dialyzate. After the resin was washed with the same buffer, PPO was eluted with the same buffer containing 1 M NaCl. The eluate was brought to 0.9 satn. with $(NH_4)_2SO_4$. After dissolving the precipitate in 20 mM NaOAc buffer, pH 4.5, the enzyme solution was dialyzed against 20 mM NaOAc buffer, pH 4.5, and the dialyzate was applied to a CM-cellulofine column. When the column was washed with 20 mM NaOAc buffer, pH 4.5, a considerable amount of protein remained on the resin, but most of the PPO was eluted. PPO in the eluate was further purified by DEAE-cellulofine column chromatography, preparative isoelectric focusing (pH, 3.5–5.0), adsorption on the membrane of a Vivapure Q Maxi H spin column, and heat treatment (65°C for 10 min). These procedures led to 150-fold purification with 4.2% recovery. The purified enzyme was homogeneous by SDS/PAGE (Fig. 1). The M_r of the enzyme was estimated to be 37 000 based on its mobility on SDS/PAGE. The isoelectric point of the purified enzyme was determined to be 4.4 by analytical gel electrofocusing.

General properties

The optimum pH of the enzyme was found to be 5.5–6.0. The optimum temperature of the enzyme was found to be 25–30°C after 30 min of incubation. After 15 min of pre-incubation with 50 mM NaOAc buffer, pH 6.0, at various temperatures, the enzyme was found to be stable at temperatures of up to 70°C. It has been reported that plant PPOs are

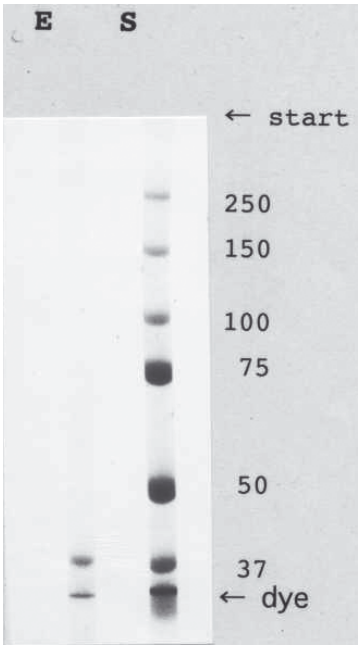

Figure 1. SDS/PAGE of purified bran PPO.
SDS/PAGE was carried out as described in experimental procedure. E, PPO; S, Precision Plus Protein Standards (Dual Color, Bio-Rad).

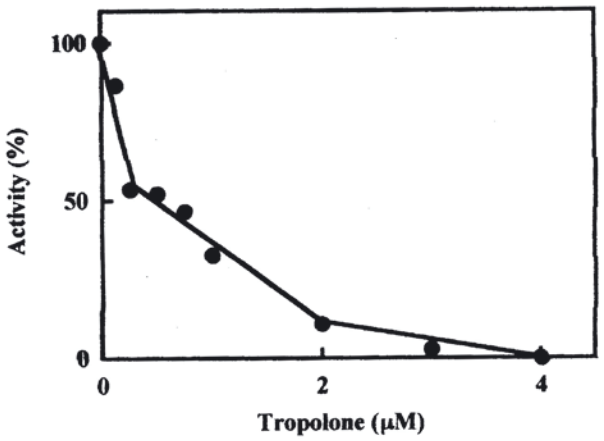

Figure 2. Effect of tropolone on PPO activity.
PPO was preincubated with tropolone at 30°C. After 5 min, L-DOPA was added and the mixture was reacted at 30°C for 30 min.

generally robust enzymes (Mazzafera & Robinson, 2000; Witisuwannakul *et al.*, 2002; Wang & Constabel, 2003). However, bran PPO is even more stable than these previously reported PPOs.

Effects of chelating reagents and SDS

Tropolone is a chelating agent and a very potent inhibitor of PPO (Wang & Constabel, 2003). Kojic acid (Chen *et al.*, 1991) has also been shown to be an effective inhibitor of PPO. When the present enzyme was incubated with tropolone and L-DOPA, tropolone completely inhibited PPO activity at a much lower concentration (2 mM) than has been reported previously (Wang & Constabel, 2003) (Fig. 2). The K_i value for tropolone was estimated to be 2.2×10^{-7} M by the method of Kahn and Andrawis (1985). The inhibitory effect of kojic acid was also potent but was lower than that of tropolone (data not shown). Therefore, PPO from wheat bran is likely to be a metalloenzyme that contains copper.

To determine if the bran PPO is a latent enzyme, the effect of SDS was tested (Fig. 3). The enzyme was not activated by SDS and indeed was inhibited at an SDS concentration above 1 mM. Moreover, PPO was extracted more effectively from bran by 20 mM phosphate buffer, pH 7.0, than by the same buffer containing some detergents or NaCl. Therefore, the PPO purified from wheat bran is not a latent enzyme, although many PPOs have been reported to be latent enzymes, which can be activated *in vitro* by detergents (Moore & Flurkey, 1990; Swain *et al.*, 1966; Wititsuwannakul *et al.*, 2002).

Substrate specificity

Various substrates were incubated with the enzyme as described in the Experimental section. The PPO showed strong affinity for caffeic acid and chlorogenic acid, although it readily oxidized caffeic acid, chlorogenic acid, pyrocathecol, 4-methylcathecol and L-DOPA. The K_m values of the PPO for caffeic acid, chlorogenic acid, pyrocatechol, 4-methyl catechol and L-DOPA as substrates were determined to be 0.077, 0.198, 1.176, 1.667 and 4.545 mM by the method of Shi *et al.* (2002).

Amino-acid sequence of the N-terminal region and the protease-inhibiting activity of PPO

The sequence of the 15 N-terminal amino-acid residues was determined to be ATDVRLSI-AHQTRFA. This sequence completely coincided with those of serpin from *Triticum aestivum* (Oster-

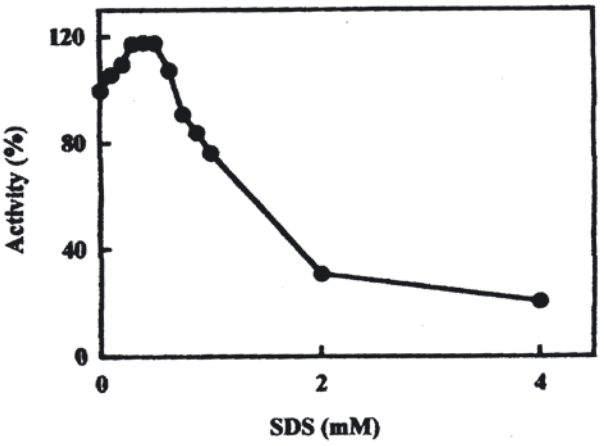

Figure 3. Effect of SDS on PPO activity.
PPO was preincubated with SDS at 30°C. After 5 min, L-DOPA was added and the mixture was reacted at 30°C for 30 min.

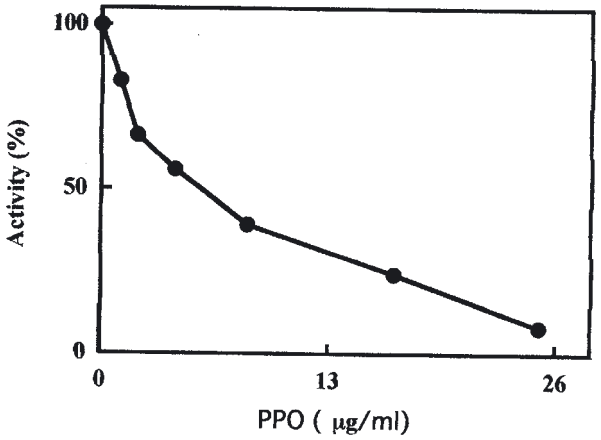

Figure 4. Effect of the purified PPO on trypsin activity.
Trypsin was preincubated with PPO purified from wheat bran at 30°C. After 5 min, MCA substrate was added and the mixture was reacted at 30°C for 10 min.

gaard *et al*., 2000; Rasmussen *et al*., 1996) and protein Z, superfamily serpin, from *Hordeum vulgare* (Nielsen *et al*., 1983; Hejgaard *et al*., 1985; Brandt *et al*., 1990). Therefore, this PPO is assumed to be a serpin. Serpins are a group of structurally related proteins, many of which inhibit serine protease activity. To confirm the above assumption, the PPO was added to a reaction mixture of trypsin, a serine protease, and assayed after incubation for 10 min at 30ºC, as described in Materials and Methods. As shown in Fig. 4, the PPO strongly inhibited trypsin activity; 50% inhibition was observed with 1.5×10^{-7} M PPO. The K_i value for PPO was estimated to be 2.3×10^{-8} M by the method of Kahn and Andrawis (1985). Therefore, the PPO is concluded to be a serpin. Serine proteases are widely distributed in animals and insects. Moreover, digestive enzymes in animals are mainly serine protease. Therefore, it is assumed that the PPO plays an important role in plant resistance against insect and herbivore damage. Since it has been suggested that PPOs are involved in plant resistance against diseases (Goy *et al*., 1992; Ray & Hammerschmidt, 1998; Mazzafera & Robinson, 2000; Li & Steffens, 2002; Shi *et al*., 2002; Witisuwannakul *et al*., 2002; Melo *et al*., 2006), this bran PPO should be a very important protein for protecting the plant against disease, virus, insect and herbivore damage.

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