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Characterization of peroxidase in buckwheat seed

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Abstract

A peroxidase (POX)-containing fraction was purified from buckwheat seed. The POX consisted of two isozymes, POX I and POX II, that were purified 6.6- and 67.4-fold, respectively. Their molecular weights were estimated to be 46.1 kDa (POX I) and 58.1 kDa (POX II) by gel filtration. While POX I and II each oxidized quercetin, *o*-dianisidine, ascorbic acid and guaiacol, only POX II oxidized ABTS. Kinetic studies revealed that POX I and II had lower K_m values for quercetin (0.071 and 0.028 mM), ABTS (0.016 mM for POX II) and ascorbic acid (0.043 and 0.029 mM) than for *o*-dianisidine (0.229 and 0.137 mM) and guaiacol (0.288 and 0.202 mM). The optimum pHs of POX I and II for various substrates were almost the same, except for quercetin; pH 8.0 for POX I and pH 4.5 for II. Their optimal temperatures were 30 °C (POX I) and 10 °C (POX II), and POX I was more stable than POX II above 30 °C.

Keywords: Buckwheat; *Fagopyrum esculentum*; Polygonaceae; Characterization; Peroxidase; Seed

1. Introduction

Plant peroxidase (EC 1.11.1.7) (POX) is widely distributed in higher plants (Van Huystee and Cairns, 1982). These enzymes are involved in a variety of functions, such as control of cell elongation (Ahmed et al., 1995), defense mechanisms (Bradley et al., 1992; Kolattukudy et al., 1992) and lignification (Blee et al., 2003). On the other hand, POX also plays important roles in food quality, including deterioration of color and flavor (Ashie et al., 1996). In soybean, aldehydes and ketones are the major contributors to 'beany' and 'green' flavors. They are mainly generated by lipid peroxidation, and the activities of enzymes such as lipoxygenase (EC 1.13.11.12) and POX are related to the generation of these flavors (Matoba et al., 1975, 1985; Anli and Tilak, 2004). In butterbur (*Petasites japonicus*), POX plays important roles in deteriorations in flavor and taste (Ibaraki et al., 1988, 1989).

Buckwheat (*Fagopyrum esculentum* Moench) is considered a healthy food. In Japan, buckwheat flour is used mainly for making noodles, and its flavor and color are important factors in its quality. However, buckwheat flour readily deteriorates (Tohyama et al., 1982; Muramatsu et al., 1986), and enzymatic activities are thought to play an important role in this deterioration (Kondo et al., 1982; Ohinata et al., 1997; Suzuki et al., 2004).

Kondo et al. (1982) partially characterized POX in buckwheat seed, and suggested that POX affects the oxidation of flavonoids in buckwheat seed. However, the characteristics of POX such as substrate specificity, thermal stability and organ distribution, which are important for clarifying the roles of POX, have not yet been determined. In this study, we purified and characterized POX in buckwheat seed.

2. Results and discussion

2.1. Purification and molecular weight of POX

POX consisted of two isozymes, POX I and POX II (Table 1), that were separated by ion-exchange chromatography (Fig. 1). We purified them 6.6- and 67.4-fold,

Abbreviations: PMSF, phenylmethyl sulfonyl fluoride; ABTS, 2,2′-azino-bis-(3-ethylthiazoline-6-sulfonate).

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Table 1
Purification of POX I and II from buckwheat seed

	Purification step	Total protein (mg)	Total activity units[a]	Specific activity (units/mg protein)	Yield (%)	Fold
	Crude extract	40,000.0	175,000[b]	4.38	100.00	1.0
	Concentration	17,400.0	182,000[b]	10.50	104.00	2.4
POX I	CM-Sepharose	70.8	321	4.53	0.18	1.0
	Sephacryl S-200	6.3	180	28.70	0.10	6.6
POX II	CM-Sepharose	97.2	12,100	124.00	6.91	28.3
	Sephacryl S-200	6.8	1990	295.00	1.14	67.4

[a] Changes in absorbance at 430 nm/min × 10^3 enzyme = 1 unit. POX activity was measured using *o*-dianisidine as a substrate.
[b] Activity was measured as a mixture of POX I and II.

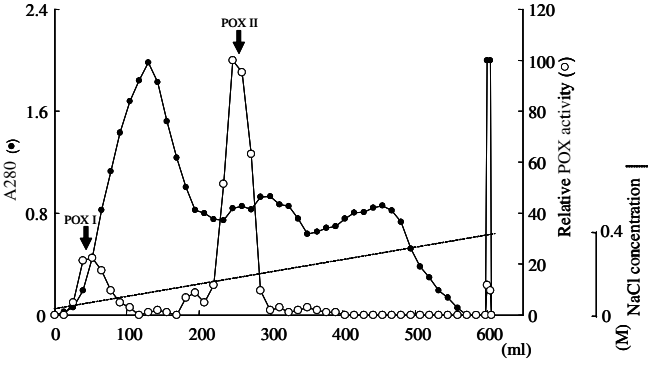

Fig. 1. Elution profile of POX activity from a CM-Sepharose column.

respectively, at a specific activity relative to crude protein extract. The yield of the enzyme was very low (0.1% for POX I and 1.14% for POX II; Table 1), and this is likely due to our purification strategy; we gave priority to the purification-fold over yield. Thus, we collected only a few fractions that had maximum activity, and discarded those around them. In this study, we purified POX from the soluble protein fraction. Kondo et al. (1982) reported that the addition of 1% (v/v) Tween 80 increased the extraction rate of POX in buckwheat flour, but we did not observe this in our experiments.

To investigate the profile of the POX isoforms in buckwheat seed, we tried both anion-exchange chromatography (DEAE-column) and cation-exchange chromatography (CM-column). We found two major peaks of POX activity in cation-exchange chromatography (Fig. 1) whereas no major peaks of POX activity were found in anion-exchange chromatography (data not shown). In addition, in each purification step, we found no POX activity except for POX I and II. These results suggest that POX I and II are the major POX in the soluble protein fraction of buckwheat seed. The molecular weights of POX I and II were 46,100 and 58,100 kDa by gel filtration. These values are similar to those of other peroxidases (Sakharov et al., 2000; Seok et al., 2001).

2.2. Kinetic constants and optimal pH of POX

The results are shown in Table 2. The K_m values for the various substrates tested (Fig. 2) were different for POX I and II. POX II had higher affinity than POX I for all of

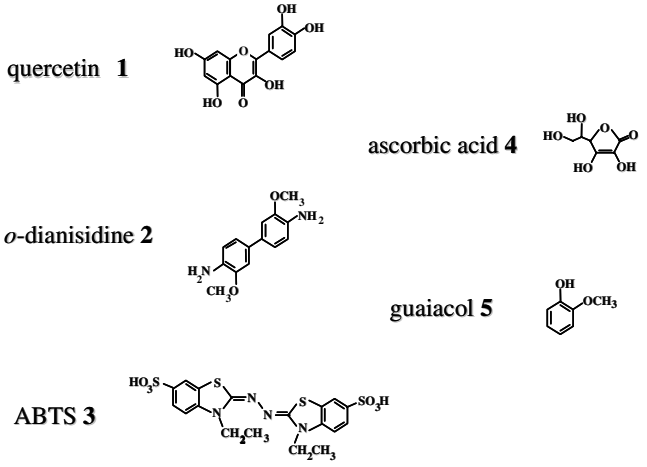

Fig. 2. Chemical structures of substrates.

Table 2
Kinetic constants and optimum pH of POX in buckwheat

	K_m (mM)		Optimum pH	
	POX I	POX II	POX I	POX II
Quercetin (**1**)[a]	0.071	0.028	8.0	4.5
o-Dianisidine (**2**)	0.229	0.137	6.0	5.0
ABTS (**3**)	n.d.[b]	0.016	n.d.	3.0
Ascorbic acid (**4**)	0.043	0.029	7.0	7.0
Guaiacol (**5**)	0.288	0.202	9.0	9.0

[a] Compound number.
[b] Not detected. Data are average of three independent experiments.

the substrates tested in this study. In particular, POX I did not react with ABTS (**3**). POX I and II had lower K_m values for quercetin (**1**), ascorbic acid (**4**) and ABTS (**3**) than for *o*-dianisidine (**2**) and guaiacol (**5**). The K_m values for *o*-dianisidine (**2**) (POX I: 0.229 mM, II: 0.137 mM) and ascorbic acid **4** (POX I: 0.043 mM, II: 0.038 mM) were similar to those of isoperoxidase PC3 from *Pleargonium graveolense* for *o*-dianisidine (**2**) (0.31 mM) and ascorbic acid (**4**) (0.03 mM). The K_m values of POX I (0.288 mM) and II (0.202 mM) for guaiacol (**5**) were lower than those of isoperoxidase PC3 from *P. graveolense* (7.3 mM) (Seok et al., 2001) and a neutral peroxidase isozyme from *Brassica napus* (3.7 mM) (Duarte-Vazquez et al., 2001). The K_m value of POX II for ABTS (**3**) (0.016 mM) was also lower than that of a neutral peroxidase isozyme from *Brassica napus* (0.7 mM) (Duarte-Vazquez et al., 2001). We propose that POX II is the major POX isozyme in buckwheat seed because more POX II was obtained than POX I (Table 1), and it has lower K_m values for various substrates than POX I (Table 2). Both POX I and II had low K_m values for phenolic substrates such as quercetin (**1**) and guaiacol (**5**). Therefore, buckwheat POX may change the color of buckwheat noodles because several reports have shown that POX plays an important role in enzymatic browning together with phenolic compounds (Kondo et al., 1982; Francisco and Juan, 2001).

The optimal pHs for various substrates were investigated. The optimal pH was different with each substrate. The optimal pH values of POX I and II were almost identical, except for quercetin (**1**). The optimal pH for quercetin (**1**) was 8.0 for POX I and 4.5 for POX II. This indicates that POX in buckwheat seeds covers a wide pH range. In buckwheat seed, most of the quercetin (**1**), rutin (quercetin 3-rutinoside) and isoquercitrin (quercetin 3-glucoside) are localized in the embryo (Suzuki et al., 2002). Their relative concentrations in the embryo were 95.5:1.1:3.4 for rutin:isoquercitrin (quercetin 3-glucoside):quercetin. Quercetin (**1**) is produced from rutin or isoquercitrin by a rutin-degrading enzyme (flavonol 3-glucosidase; Suzuki et al., 2002) (rutin degrading enzyme; Yasuda and Nakagawa, 1994), which is also localized in the embryo (Suzuki et al., 2002). In this study, the major POX activity was also localized in the embryo (Fig. 3). Quercetin (**1**) acts as a substrate of guaiacol peroxidase, and the anti-fungal agent 3,4-dihydroxybenzoic acid is formed by the peroxidase-dependent oxidation of quercetin (Takahama and Hirota, 2000). Based on these results, we propose that POX plays important roles in antioxidant activity and the production of an anti-fungal agent when quercetin is used as a substrate in buckwheat seed.

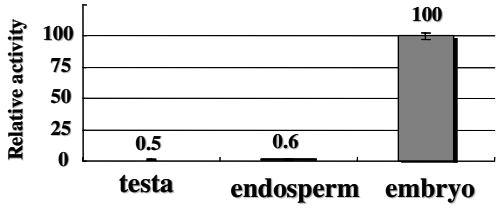

Fig. 3. Organ distribution of POX activity in the fully ripe seed. POX activity was measured using 20 seed embryo, testa or endosperm. POX activity was measured using *o*-dianisidine (**2**) as a substrate (see Section 3). Data are means of three independent experiments. Bar indicates SD.

2.3. Optimal temperature and thermal stability of POX

The optimal temperature and thermal stability of POX activity were investigated using *o*-dianisidine (**2**) as a substrate (Fig. 4). The optimal temperature for POX I was 30 °C whereas it was 10 °C for II. More than 50% of the activity of POX I was retained in the temperature range of 0–50 °C. On the other hand, POX II showed high activity at a lower temperature range of 0–20 °C, and its activity decreased gradually at more than 20 °C.

The thermal stabilities of POX I and II were also different. POX I is more stable against higher temperature than POX II. POX I was stable at 0–30 °C and unstable above 40 °C, whereas POX II was stable at 20 °C and unstable above 30 °C. POX I and II were inactivated at 60 and 50 °C, respectively.

2.4. Effect of various compounds on the peroxidative activity of POX

To characterize POX I and II, the effects of various compound were examined (Table 3). In isoperoxidase PC3 from *P. graveolense* (Seok et al., 2001), peroxidase activity was boosted 7-fold by imidazole and 4-fold by adenine when *o*-dianisidine (**2**) was used as a substrate. However,

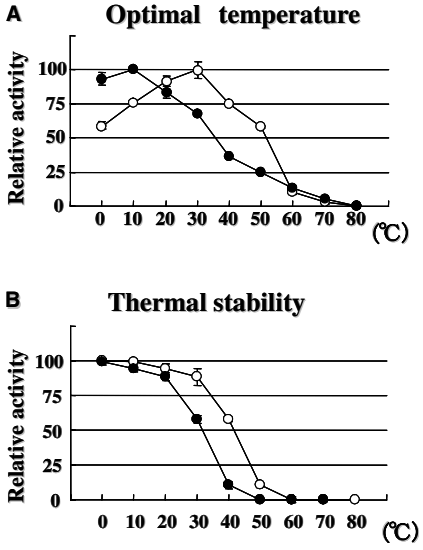

Fig. 4. Optimum temperature and thermal stability of POX. (A) Optimum temperatures of POX I (○) and II (●). POX activity was measured at the optimum pH of each POX isozyme using *o*-dianisidine (**2**) as a substrate (see Section 3). Data are means of three independent experiments. Bar indicates SD. (B) Thermal stabilities of POX I (○) and II (●). POX activity was measured at the optimum pH of each POX isozyme using *o*-dianisidine (**2**) as a substrate (see Section 3). Data are means of three independent experiments. Bar indicates SD.

Table 3
Effect of various compounds on peroxidative activity of POX

Effector	Nitrogenous compounds						Free-fatty acid					
	Ammonium ion		Imidazole		Histidine		Linolenic acid		Linoleic acid		Oleic acid	
	POX I	POX II	POX I	POX II	POX I	POX II	POX I	POX II	POX I	POX II	POX I	POX II
Substrate												
Quercetin (**1**)[a]	98.8	93.5	27.7	75.9	28.8	56.8	83.9	65.1	97.8	24.0	37.6	28.4
o-Dianisidine (**2**)	72.7	147.8	2.5	18.5	5.4	87.0	78.7	63.4	68.4	51.8	80.9	58.7

Data are expressed relative to the POX activity without nitrogenous compounds or free-fatty acid which was given a value of 100.
Data are average of three independent experiments.
[a] Compound number.

in this study, among various compounds, only ammonium ion increased the activity of POX II when *o*-dianisidine (**2**) was used as a substrate. In contrast, imidazole and histidine strongly inhibited the activities of POX I when *o*-dianisidine (**2**) was used as a substrate. In addition, POX II tends to be inhibited by free-fatty acid compared with POX I.

Based on these results, we can perform a selective assay of POX I and II in crude protein extract spectrophotometrically as follows: to measure only POX I, measure the activity at pH 8.0 using quercetin as a substrate with linoleic acid; to measure only POX II, measure the activity using ABTS (**3**) as a substrate. The selective assay of POX isozyme may be useful for clarifying the roles of POX in food quality or for physiological analysis.

3. Experimental

3.1. Plant materials

A common buckwheat variety (*Fagopyrum esculentum* Moench var. Kitawasesoba) was grown at the experimental field of the National Agricultural Research Center for the Hokkaido Region in Memuro, Hokkaido, Japan (latitude: 42°53′ longitude: 143°03′). Buckwheat seeds were sown on June 6th in 2001 and harvested in late August. Harvested seeds were dried at 40 °C for about 1 week, and then threshed and stored at 4 °C until used for experiments.

3.2. Purification of POX

Buckwheat seeds were milled using a mortar after the removal of panicles. Buckwheat flour (200 g fresh weight) was homogenized with 2000 ml of extraction buffer containing 50 mM acetate-NaOH buffer (pH 5.0), 1 mM EDTA and 1 mM fresh PMSF for 1 h. A crude enzyme solution was obtained by centrifugation and then precipitated with 0–80% saturation of solid $(NH_4)_2SO_4$. To increase the purification-fold, the solution was divided into eight portions before all the steps below were carried out. The precipitate was dissolved in buffer A, which contained 50 mM acetate-NaOH buffer (pH 5.0), and dialyzed overnight against buffer A. The dialyzed enzyme solution was applied to a CM-Sepharose column (2.4 × 11.5 cm, Amersham Pharmacia Biotech) equilibrated with buffer A. The POX was eluted using a linear 600-ml gradient of 50–400 mM NaCl in buffer A. Active fractions were collected and loaded onto a Sephacryl S-200 column (2.4 × 66 cm, Amersham Pharmacia Biotech) equilibrated with buffer A. Active fractions were collected and stored at −30 °C. In each purification step, POX activity was measured using *o*-dianisidine (**2**) as a substrate. All of the above steps were carried out at 4 °C.

3.3. Assay of in vitro POX activity

POX activities were assayed by a modification of the procedure described by Amako et al. (1994) and Yamasaki et al. (1997). POX activities were determined at 22 °C by measuring the initial rate of the decrease in absorbance at 370 nm (quercetin (**1**)) and the increase in absorbance at 430 nm (*o*-dianisidine (**2**)), 415 nm (ABTS (**3**)), 290 nm (ascorbic acid (**4**)) and 475 nm (guaiacol (**5**)). The assay mixture contained 200 mM buffer (buffer at optimal pH for POX activity of each substrate), 0.3 mM H_2O_2, substrate (0.3 mM quercetin (**1**), 1 mM *o*-dianisidine (**2**), 0.2 mM ABTS (**3**), 1 mM ascorbic acid (**4**) or 1 mM guaiacol (**5**)) and 50 μl of enzyme solution (about 0.5 ng protein) in a total volume of 1 ml.

3.4. Determination of optimal pH, optimal temperature, thermal stability and K_m value of enzyme activity

The enzyme activity was determined at different pHs, of 2.0 (200 mM glycine–HCl buffer), 3.0–4.0 (200 mM citrate–NaOH buffer), 5.0–6.0 (200 mM acetate–NaOH buffer), 7.0 (200 mM phosphate–NaOH buffer), 8.0 (200 mM Tris–HCl buffer), or 9.0–10.0 (200 mM Borate–NaOH buffer), at 22 °C using *o*-dianisidine (**2**) as a substrate.

POX activity was measured at 0–80 °C using *o*-dianisidine (**2**) as a substrate. Reaction mixtures were pre-incubated at each temperature for 30 min prior to measuring the POX activity.

The enzyme solution was incubated at 0–80 °C for 4 h prior to assay, and then POX activity was assayed at

22 °C using *o*-dianisidine (**2**) as a substrate. The K_m values were determined by Lineweaver–Burk plots at different concentrations of each substrate ranging from 0.005 to 0.3 mM (quercetin (**1**)), 0.006–1 mM (*o*-dianisidine (**2**)), 0.002–0.2 mM (ABTS (**3**)), 0.006–4 mM (ascorbic acid (**4**)) and 0.01–6 mM (guaiacol (**5**)).

3.5. Effect of various compounds on the peroxidative activity of POX

To investigate the effect of various compounds on the peroxidative activity of POX, 0.18 mM nitrogenous compounds (according to a minor modification of Seok et al. (2001)) or 3.6 mM free-fatty acids (dissolved in ethanol) were added to the assay mixture, which contained substrate (0.3 mM quercetin (**1**) or 1 mM *o*-dianisidine (**2**)), 200 mM buffer (buffer in optimum pH of POX activity for each substrate), 0.3 mM H_2O_2 and 50 μl of enzyme solution (about 0.5 ng protein) in a total volume of 1 ml. As a control, reaction mixture without nitrogenous compounds or free-fatty acid was used.

3.6. Protein determination

Total soluble protein concentrations were measured by the method of Bradford (1976) using bovine serum albumin is a standard.

3.7. Estimation of molecular weight of POX

Gel filtration was carried out using the purified POX isozyme. Purified POX was loaded onto a Sephacryl S-200 column (2.4 × 66 cm, Amersham Pharmacia Biotech) equilibrated with buffer A. The molecular weight was determined using a standard curve of elution volume vs. log MW derived from standard proteins.

3.8. Organ distribution of POX activity in buckwheat seed

Embryo, testa and endosperm of 20 buckwheat seeds were separated carefully using tweezers after removal of the panicles. Soluble protein was extracted from the separated organs in buffer A, and POX activity was Measured using *o*-dianisidine (**2**) as a substrate as described in Section 3.3.

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