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Effects of metal ions on the activity and stability of peroxidase in Tartary buckwheat shoots

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ABSTRACT

This is the first report to purify and characterize POX in shoots of buckwheat species. POX was partially purified from Tartary buckwheat shoots by 48.0 fold with a final yield of 9.07%. During ion-exchange and gel filtration chromatography, only one peak corresponding to POX activity was found. The molecular weight of POX was determined to be 37.5 kDa using gel filtration chromatography. The optimal pH of POX activity was 5.5 (guaiacol, quercetin) and 5.0 (ABTS). The K_m of POX activity was 22.3 mM (guaiacol), 6.3 mM (ABTS) and 0.92 mM (quercetin). In contrast, the K_m for quercetin in the presence of Fe^{3+} ions was two orders of magnitude less (0.018 mM) than that in its absence. The stability of POX activity was increased in the presence of trivalent metal ions, even after 186 h in solution. POX activity was retained by 83.6% and 56.1% in the presence of 1 mM Fe^{3+} and Al^{3+} ions, respectively, whereas it was completely inactivated in their absence. To the best of our knowledge, this is the first study to detail the activation and stabilization of POX activity in relation to trivalent metal ions.

Keywords: Tartary Buckwheat; Characterization; Peroxidase; Metal Ion; Quercetin; K_m

1. INTRODUCTION

Buckwheat is a rich source of functional polyphenols such as rutin and quercetin [1-4]. Tartary buckwheat leaves contain rutin and anthocyanins in high concentrations (rutin: ca 100 mg/g DW, anthocyanin: ca 0.8 mg/g DW) [5,6]. For plants, the physiological roles of polyphenols are reported to provide UV-B protection and have antimicrobial properties [7]. Although some physiological roles of polyphenols, including UV screening, anti-desiccation and anti-cold stresses [5], related to stress resistance have been studied in buckwheat, the roles of polyphenols still remain unclear.

On the other hand, plants have polyphenol-oxidizing enzymes such as peroxidase, which is related to lignification [8] and resistance against infection by pathogens. Tartary buckwheat (*Fagopyrum tataricum*) shoots contain a substantial amount of peroxidase (POX) activity, the characteristics and physiological roles of which have not been studied. Peroxidases are classified by various categories on the basis of substrate specificity and comparison of amino acid sequences. For example, ascorbate peroxidase (APX, EC 1.11.1.11) and plant peroxidase (POX, EC 1.11.1.7) are heme-containing proteins and glutathione peroxidase (GPX, EC 1.11.1.9) is a protein that forms oxidized glutathione, which is related to antioxidants and detoxification. POXs are considered to have different properties in different tissues [8]. Therefore, characterization of POX is important in order to understand the physiological roles of polyphenols in buckwheat leaves.

POX has been partially purified and characterized in both common (*F. esculentum*) and Tartary buckwheat seeds [9,10]. These reports revealed that POX consists of at least two isozymes and has a lower K_m for phenolic substrates such as quercetin and guaiacol than other substrates. However, to the best of our knowledge, the purification and characterization of POX in buckwheat shoots has not yet been reported in buckwheat species. Some papers have described POX as being activated by Ca^{2+}, Fe^{3+} or Al^{3+} ions [21,22]. Some kinds of POX pro-

tein have been reported to contain metal ions, but Ca^{2+} sometimes inhibited POX activities [23]. These reports revealed the importance concerning the effects of metal ions on POX activity in studying the physiological roles of POX. Therefore, we investigated the effects of metal ions on purified POX activities as well.

2. MATERIALS AND METHODS

2.1. Plant Material

Tartary buckwheat (*F. tataricum* var. Hokkai T10) was grown in the experimental field at the National Agricultural Research Center for the Hokkaido Region in Memuro, Hokkaido, Japan. On June 7th, 2012, shoots grown to 5 days after germination were collected and stored at −80˚C until they were used.

2.2. Extraction and Purification of POX

Buckwheat shoots (20 g fresh weight) were homogenized with 200 ml of extraction buffer containing 50 mM acetate-LiOH buffer (pH 5.5) and 0.3% TritonX-100 (v/v) for 30 min according to the method in previous report [11]. A crude enzyme solution was obtained by centrifugation and then precipitated with 0% - 80% saturation of solid $(NH_4)_2SO_4$. The precipitate was dissolved in buffer A, which contained 50 mM acetate-LiOH buffer (pH 5.5), and dialyzed. The dialyzed enzyme solution was applied to a 2.4 × 7.0 cm SP-Sepharose column (GE healthcare Japan cooperation; Asahigaoka, 191-0065 Hino Tokyo Japan) and equilibrated with buffer A. POX was eluted with a linear gradient of 50 - 500 mM LiCl in the same buffer. Active fractions were collected and further purified by gel filtration chromatography on a 1.5 × 55 cm Sephacryl S-200 column (GE healthcare Japan cooperation) with equilibrated buffer A, which contained 150 mM LiCl, and eluted with the same buffer. All of the above steps were carried out as soon as possible at 4˚C. In each purification step, POX activity was measured using guaiacol as the substrate. After gel filtration, POX activity became unstable, and did not improve, compared with cation exchange chromatography, following a subsequent purification fold. Therefore, we tried to stabilize POX by adding both $FeCl_3$ and $AlCl_3$. However, addition of these ions caused a decrease in performance after gel filtration (data not shown). We then decided to add $FeCl_3$ or measure enzymatic properties as soon as possible after active fractions were obtained by gel filtration. Using guaiacol as substrate, one unit of POX activity was defined as the amount of enzyme which increased absorbance by 1.0 min^{-1} at 490 nm. Total protein content was measured using bovine serum albumin (BSA) as the standard protein.

2.3. Assay of POX Activity

POX was assayed according to the method of previous report [11]. POX activities were determined at 25˚C by measuring the initial rate of the increase in absorbance at 490 nm (guaiacol) and 415 nm (ABTS), and the decrease in absorbance at 370 nm (quercetin). The assay mixture contained 50 mM buffer at optimal pH, 9.3 mM H_2O_2, 85 mM guaiacol 87 mM ABTS and 0.3 mM quercetin as the substrate.

2.4. Estimation of Molecular Weight of POX

Gel filtration chromatography was used to estimate the molecular weight of POX according to the method in previous report [11]. A POX solution was loaded on a 1.5 × 55 cm Sephacryl S-200 column with equilibrated buffer A, which contained 150 mM LiCl. The molecular weight was determined using a calibration curve derived from the following standard proteins: *β*-amylase (200 kDa), BSA (66 kDa), carbonic anhydrase (29 kDa), and cytochrome C (12.4 kDa).

2.5. Determination of Optimal pH and K_m

Using guaiacol, ABTS and quercetin as substrates, POX activity was determined under the following pH levels at 25˚C: 2.0 (50 mM glycine-HCl buffer); 3.0 - 4.0 (50 mM citrate-LiOH buffer); 5.0 - 6.0 (50 mM acetate-LiOH buffer); 7.0 (50 mM phosphate-LiOH buffer); 8.0 (50 mM Tris-HCl buffer); and 9.0 - 10.0 (50 mM borate-LiOH buffer). K_m was determined by Lineweaver-Burk plots at different concentrations of each substrate. The substrate mixture contained 1 mM $FeCl_3$.

2.6. Effect of Various Metal Ions on Purified POX Activity

After the gel filtration step, purified POX was incubated for 0 h, 96 h and 186 h at 4˚C with 1 mM of various metal chlorides ($FeCl_3$, $AlCl_3$, $CaCl_2$, $MnCl_2$, $MgCl_2$, KCl and NaCl), and POX activity was measured using guaiacol as a substrate. Assay was performed according to the method of previous report [11].

3. RESULTS AND DISCUSSION

3.1. Purification of Peroxidase from Tartary Buckwheat Shoots

POX in Tartary buckwheat shoot was purified by ammonium sulfate precipitation, cation-exchange chromatography and gel filtration (**Table 1**). We found one large peak corresponding to POX activity on cation-exchange chromatography (**Figure 1**). In comparison, buckwheat seeds contain at least two peaks corresponding to POX

Table 1. Purification of the POX in Tartary buckwheat shoots.

Purification step	Total protein (mg)	Total activity units[a]	Specific activity (units/mg protein)	Yield (%)	Fold
Crude exrtract	109	6390	58.7	100	1.00
Ammonium sulfate precipitation	44.3	5910	133	92.5	2.30
Cation exchange	0.0374	1130	2850	17.7	48.6
Gel filtration	0.0205	579	2820	9.07	48.0

[a]Changes in absorbance at 490 nm/min × 10^3 enzyme = 1 unit.; POX activity was measured using guaiacol as a substrate.

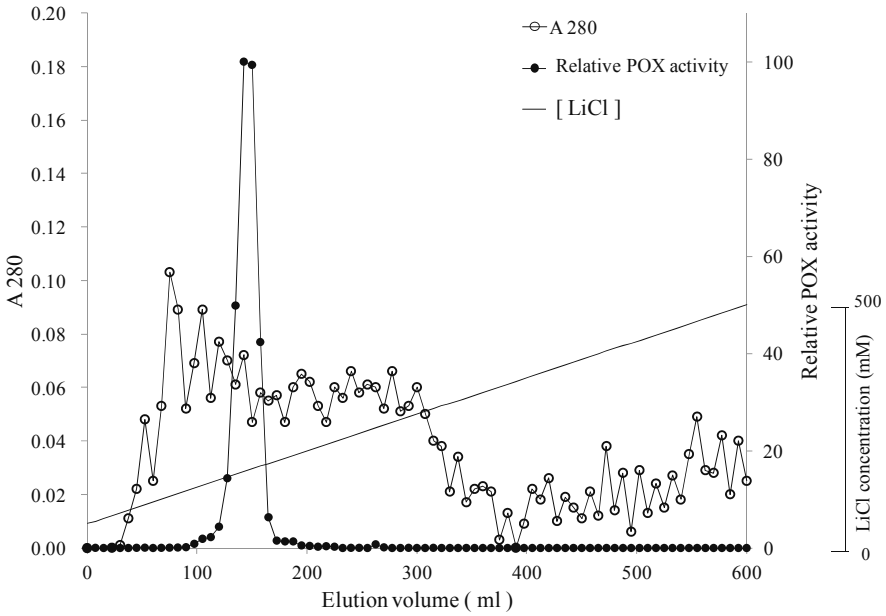

Figure 1. Elution profile of POX activity on the SP-sepharose column

activity [3,10]. Therefore, the composition of cationic POX isozymes in Tartary buckwheat shoots must be different from that found in common and Tartary buckwheat seeds. Slight POX activity was also found on anion-exchange chromatography (data not shown), even though anionic POX was too weak to purify. It reported that POX isozymes induced by UV-B radiation in buckwheat seeds differed from those induced in leaves [9]. These results suggest that POX may serve different roles in seeds compared with shoots. Based on gel filtration chromatography, the molecular weight of POX from buckwheat shoots was estimated to be 37.5 kDa. This value was smaller than that of POX from both common (46.1 kDa) [10] and Tartary buckwheat seeds (46.8 kDa) [11], but similar to that of both *Allium sativa* L. (37.8 kDa) [12] and *Withania somnifera* (34 kDa) [13].

3.2. Optimal pH of Purified POX

The optimal pH of purified POX was investigated using guaiacol, ABTS and quercetin as substrates, and found to be similar to each substrate used in this study (**Table 2**). The optimal pH for guaiacol was 5.5. This value was similar to that of peroxidase from both *Spinacia oleracea* leaves (pH 5.2) [14] and *Brassica napus* L. (pH 5.5) [15]. POX activity decreased with either an increase or a decrease of pH. The optimal pH for guaiacol in common buckwheat seed was 9.0, which was different from that in POX from Tartary buckwheat shoots [10]. On the other hand, the optimal pH for guaiacol in Tartary buckwheat seed was 6.0, which was similar to that of POX in Tartary buckwheat shoots for guaiacol [11]. The optimal pH of POX in Tartary buckwheat shoots for ABTS was 5.0. This value was similar to those of peroxidase from *Cucumis melo* L. (pH 5.5) [16] and *Capsicum annuum* L. (pH 4.5) [17]. The optimal pH for quercetin was 5.5, which was higher than that of peroxidase from both *Vitis vinifera* and *Allium sativum* L. [12,18]. When quercetin was used as a substrate, POX could retain activity in a wider pH range compared to guaiacol and ABTS. Buckwheat shoots, and especially leaves and cotyledon, contain a large amount of rutin [3,19] and exhibit rutinosidase activity [19]. When these structures are damaged by UV and cold stress, quercetin is generated from rutin via rutinosidase activity [3]. Therefore, a wider pH range of POX activity against quercetin may relate to quercetin metabolism *in vivo*.

Table 2. Optimum pH of purified POX.

pH	Relative activity (%)[a]		
	Guaiacol	ABTS	Quercetin
2.0	n.d.[b]	56.8 ± 8.0	14.0 ± 0.7
3.0	n.d.	5.6 ± 2.8	39.4 ± 0.6
4.0	34.0 ± 1.0	48.3 ± 1.6	88.2 ± 2.7
5.0	78.2 ± 1.0	100 ± 4.4	94.1 ± 4.0
5.5	100 ± 1.2	55.2 ± 1.4	100 ± 0.2
6.0	50.4 ± 1.5	26.7 ± 0.9	95.0 ± 2.4
7.0	n.d.	12.2 ± 1.8	83.5 ± 1.6
8.0	1.3 ± 0.3	n.d.	35.5 ± 0.1
9.0	1.7 ± 0.5	n.d.	24.9 ± 0.3
10.0	0.8 ± 0.2	n.d.	12.9 ± 1.4

[a]Guaiacol and quercetin: pH 5.5 = 100%,; ABTS: pH 5 = 100%.; [b]Not detected.; Date are means ± SD (n = 3).

This reinforces the idea that the anti-fungal agent 3,4-dihydroxybenzoic acid is formed by peroxidase-dependent oxidation of quercetin on browning onion scales [20].

3.3. Effect of Metal Ions on Purified POX Activity

After gel-filtration chromatography, the effects of various metal ions on purified POX activity were investigated at 0 h, 96 h and 186 h in storage (**Table 3**). At 0 h, POX activity ranged between 93.8% ($CaCl_2$) to 127% ($FeCl_3$) compared to the activity without metal ions (= defined as 100%). After storage at 96 h and 186 h, POX did not retain activity in either the absence or presence of divalent and monovalent metal ions. On the other hand, when $FeCl_3$ or $AlCl_3$, where added POX activity was retained even after storage at 186 h at 83.6% ($FeCl_3$) and 56.1% ($AlCl_3$). These results suggest that POX in Tartary buckwheat shoots can be stabilized by trivalent metal ions. In addition, it suggests that $FeCl_3$ stabilizes POX more effectively than $AlCl_3$. Numerous reports have shown that POX is activated by divalent ions such as Ca^{2+} ions [21,22]. On the other hand, only a few studies have reported that POX is activated by both Fe^{3+} [23] and Al^{3+} [24]. In addition, in these reports, POX activities were activated only about 2.1 to 3.3 folds. However, no studies have found that the stability of POX is enhanced in the presence of trivalent metal ions. Therefore, our finding reveals novel characteristics concerning POX and suggests the importance of studying its physiological roles in plants.

3.4. Kinetics of Purified POX

We measured the K_m of POX both in the presence and absence of $FeCl_3$ and $AlCl_3$ using guaiacol, ABTS and quercetin as substrates. The results are summarized in **Table 4**. In the absence of the trivalent ions, K_m values

Table 3. Effect of metal ions on POX activity.

Compounds	Relative activity (%)[a]		
	0 h	96 h	186 h
Non	100[b] ± 2.2	n.d.	n.d.
$FeCl_3$	127 ± 2.4	118.5 ± 0.5	83.6 ± 2.7
$AlCl_3$	102 ± 4.0	108.8 ± 0.3	56.1 ± 2.7
$CaCl_2$	93.8 ± 10.3	n.d.	n.d.
$MnCl_2$	99.6 ± 8.3	n.d.	n.d.
$MgCl_2$	100 ± 17.8	n.d.	n.d.
KCl	108 ± 8.6	n.d.	n.d.
NaCl	106 ± 2.4	n.d.	n.d.

[a]Assay was carried out using guaiacol as a substrate.; [b]Activity measured at 0 h in metal ion free condition is 100%.; Date are means ± SD (n = 3).

Table 4. K_m of purified POX.

Substrate	K_m (mM)		
	-[a]	1 mM $FeCl_3$	1 mM $AlCl_3$
Guaiacol	22.3	16.4	7.50
ABTS	6.30	0.86	11.5
Quercetin	0.92	0.018	19.1

[a]Absence of trivalent metal ions.

were 22.3 mM, 6.3 mM and 0.92 mM for guaiacol, ABTS and quercetin, respectively. Compared to POX in other plants, the K_m for guaiacol was higher than that from *Jastropha curcas* leaves (0.17 mM) [25] and *Brassica napus* roots (3.7 mM) [15]. The K_m for ABTS was also higher than that from *Eupatrium odoratutum* (0.12 mM) [26] and *Brassica napus* roots (0.7 mM) [15]. In addition, the K_m for quercetine was much higher than that from both *Allium sativum* L. (0.033 mM) [12] and *Vicia faba* leaves (0.023 mM) [27]. Furthermore, the presence of $FeCl_3$ enhanced the affinity of POX, resulting in a decreased K_m of 16.4 mM, 0.86 mM and 0.018 mM for guaiacol, ABTS and quercetin, respectively. The presence of $FeCl_3$ led to a dramatic decrease of K_m, about 51.1 fold, for quercetin compared to its absence, which, when compared to the K_m for quercetin in other plants, is one of the lowest values so far reported in: both common buckwheat seed (0.028 mM) [10] and *Vicia faba* leaves (0.023 mM) [27]. On the other hand, the presence of $AlCl_3$ increased the K_m for quercetin about 20 fold compared to K_m in its absence. These results suggest that POX has different mechanisms for controlling its activity in relation to both $FeCl_3$ and $AlCl_3$. Based on these results, we suggest that POX in Tartary buckwheat shoots have unique characteristics, and novel aspects related to the physiological roles of POX that warrant for further investigation.

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ABBREVIATIONS

ABTS: 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid);
DW: dry weight.