Pergamon

0031-9422(95)00098-4

Phytochemistry, Vol. 39, No. 5, pp. 981–984, 1995

0031-9422/95 $9.50 + 0.00

ACIDIC PEROXIDASES FROM SUSPENSION-CULTURES OF SWEET POTATO

SANG-SOO KWAK,* SOO-KYUNG KIM,*† MOON-SOON LEE,* KYUNG-HEE JUNG,* IL-HYUN PARK† and JANG-RYOL LIU*

*Bioresources Research Group, Genetic Engineering Research Institute, KIST, P.O. Box 115, Yusong, Taejon 305-600, Korea; †Department of Biochemistry, Chungnam National University, Yusong, Taejon 305-764, Korea

(*Received in revised form* 4 *January* 1995)

Key Word Index—*Ipomoea batatas*; Convolvulaceae; suspension culture; peroxidase; pyrogallol.

Abstract—We established an efficient production system of peroxidase (POD, EC 1.11.1.7) in cultured cells of sweet potato [*Ipomoea batatas* (L.) Lam. cv White Star]. A cell line SP-47 derived from cell suspension cultures of sweet potato produced a level of POD, i.e. *ca* 6800 units g^{-1} dry cell wt (substrate, pyrogallol) when 1 g (fr. wt) of cells was inoculated into 50 ml LS medium supplemented with 1 mg l^{-1} 2,4-D in a 300 ml conical flask and cultured at 25° in the dark (100 rpm) for 25 days. Three acidic POD isoenzymes (A-1, A-2 and A-3) were purified to homogeneity from suspension-cultured cells by DEAE-cellulose and gel filtration column chromatography. A major isoenzyme A-2, a monomeric hemoprotein (M_r *ca* 40000, p*I*: *ca* 4.5, RZ value: 4.1), occupied more than 7.5% of total protein in cultured cells and *ca* 88% of total POD activity. The K_m values of isoenzyme A-2 for pyrogallol and H_2O_2 were 2.53 and 5.76 mM, respectively. RZ values for two minor isoenzymes A-1 (M_r *ca* 43000, p*I* *ca* 5.0) and A-3 (M_r *ca* 40000, p*I* *ca* 6.0) were 1.9 and 1.6, respectively, which was quite low compared with isoenzyme A-2.

INTRODUCTION

Peroxidase (POD, EC 1.11.1.7) is a ubiquitous enzyme that reduces H_2O_2 in the presence of electron donor. POD is widely used as an important reagent for clinical diagnosis and microanalytical immunoassay because of its high sensitivity in reaction [1, 2]. New applications for POD have been suggested in the field of medicinal, chemical and food industry including elimination of phenolic and aromatic compounds in waste treatment [3] and practical use for this enzyme have been explored in recent years. So far, the major source of commercially available POD is intact roots of horseradish (*Armoracia rusticana*), although it is found in a vast number of plants and microorganisms [4].

POD activity in plants increases in response to a variety of stress including viral, microbial, and fungal infections, salt stress, wounding or air pollution [5–8]. Cultured plant cells are considered to be grown under high stress conditions [9], which suggests that plant cell cultures are an efficient system for POD production. Thus, plant cell and hairy root cultures have been studied for the production of POD: horseradish [10–12], peanut [13, 14], radish [15], tobacco [16]. However, there is no cell culture system to substitute horseradish roots because of their low productivity. In the previous paper, we selected a sweet potato cell line with the highest yield of POD among 41 cell lines derived from 25 plant species [17]. In this report, we describe the purification and characterization of major POD isoenzymes produced in suspension cultures of sweet potato (*Ipomoea batatas*).

RESULTS AND DISCUSSION

POD activity and protein content during cell culture

The cell growth of sweet potato was maximum on 15 days after subculture (DAS) showing a typical sigmoidal growth curve: there was an initial lag period (up to 8 DAS) followed by an exponential rise (Fig. 1A). However, POD activity per g dry cell wt showed a progressive deceleration prior to a linear increase from 12 to 25 DAS, resulting in *ca* 7 days interval between the two exponential rises in the cell growth and POD activity. These trends did not agree with those of other cell cultures showing growth concurrent with POD activity [11, 12, 15]. The POD activity at 25 DAS was *ca* 6800 units, *ca* 30 times higher than that of intact roots of horseradish plants grown in a greenhouse [17].

The protein content per g dry cell wt maintained almost constant unit 25 DAS and then decreased with further culture, whereas the POD specific activity (unit mg^{-1} protein) continuously increased from 12 DAS to the end of cultures (Fig. 1B). The POD isoenzyme patterns were almost the same, regardless of cell growth stage, but some acidic isoenzymes slightly increased after 25 DAS (data not shown). These results indicate that the

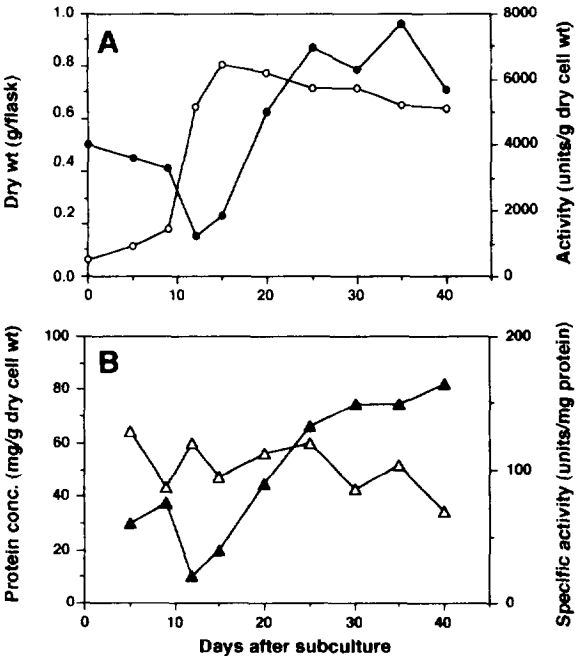

Fig. 1. Changes of POD activity and protein content in cell suspension cultures of sweet potato in LS basal medium supplemented with 1 mg l^{-1} 2,4-D and 30 g l^{-1} sucrose. (A) Time course of cell growth and POD activity on the basis of g dry cell wt; (B) time course of protein content (mg protein g^{-1} dry cell wt) and POD specific activity (unit mg^{-1} protein). —○—: dry wt (g/flask); —●—: D POD activity (units g^{-1} dry wt); —△—: protein concentration (mg g^{-1} dry wt); —▲—: specific activity (units mg^{-1} protein).

POD activity in suspension cultures of sweet potato is closely associated with cell growth and culture stresses derived from subculture (dilution effect) and medium depletion.

Enzyme purification

Three acidic POD isoenzymes, A-1 (frs 79–82), A-2 (frs 84-87) and A-3 (frs 90–93), were separated from suspension-cultured cells by DEAE-cellulose column chromatography (Fig. 2). A major isoenzyme A-2 was purified to homogeneity by this step. The specific activity

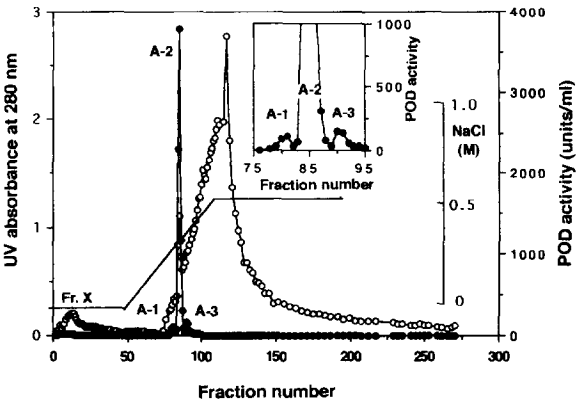

Fig. 2. DEAE-cellulose elution profile. *Ca* 230 mg protein was applied to the column and eluant was collected in 10 ml (flow rate 1 ml min^{-1}), of which 10 µl each was assayed for the activity. The chromatographic conditions are described in the Experimental. —●—: POD activity (units m^{-1}); —○—: protein elution profile (UV at 280 nm); —: NaCl gradient.

(activity mg^{-1} protein) of two minor isoenzymes A-1 and A-3 were increased by further purification using Sephadex G-100 column chromatography (Table 1). On the basis of the purification by DEAE-cellulose, a major isoenzyme A-2 occupied more than 7.5% of total protein in cultured cells and was *ca* 88% of total POD activity. The POD activity in cells was *ca* 96% of total activity in flask, whereas that secreted in the medium was *ca* 4% (data not shown). Fraction X (frs 4–13) had a small amount of a putative basic POD activity (*ca* 1.3% of total activity). To our knowledge, the content of a major isoenzyme A-2 in cultured sweet potato cells demonstrated in this study is at a significantly higher level compared with peanut cells, occupying 3% of total protein, which may be the highest level among plant cell lines reported [14].

Properties of purified isoenzymes

Major properties of three isoenzymes purified from suspension-cultured cells of sweet potato are shown in Table 2. The M_rs of three isoenzymes were estimated to

Table 1. Purification of three POD isoenzymes from suspension-cultured cells of sweet potato

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units mg^{-1} protein)	Yield (%)	Purification (-fold)
Crude extract from cells	231	67000	290	100.0	1.00
DEAE-cellulose					
Fraction X	1.47	543	369	0.8	1.27
A-1	3.96	2230	562	3.3	1.94
A-2	19.2	35500	1850	53.0	0.40
A-3	7.22	2068	287	3.1	0.99
Sephadex G-100					
A-1	2.31	1450	627	2.2	2.16
A-2	17.4	26000	1490	39.1	5.14
A-3	2.14	1330	622	2.0	2.14

Table 2. Properties of three POD isoenzymes purified from suspension-cultured cells of sweet potato

	Isoenzyme A-1	Isoenzyme A-2	Isoenzyme A-3
M_r	43000	40000	40000
Isoelectric point	5.0	4.5	6.0
RZ value	1.9	4.1	1.6
Optimum pH	7.5	7.1	7.1
Optimum temp	37	37	37
K_m values (mM)			
for pyrogallol	5.70	2.53	7.14
H_2O_2	2.65	5.76	2.78

be 40000 for isoenzymes A-2 and A-3, 43000 for isozyme A-1 by SDS–PAGE (Fig. 3) and HPLC for gel filtration chromatography. The three isoenzymes showed an acidic POD with isoelectric point (p*I*) ranging from 4.5 to 6.0 and had an *A* maximum at 403 nm. Particularly, RZ value (A_{403}/A_{280}) which is an expression of the ratio of hemin to protein content was quite high for a major isoenzyme A-2 (4.5) compared with those of the minor isoenzymes A-1 (1.9) and A-3 (1.6). These results suggest that the three isoenzymes from suspension-cultured cells of sweet potato are similar to the monomeric acidic hemoprotein of other plant PODs, such as horseradish POD [6].

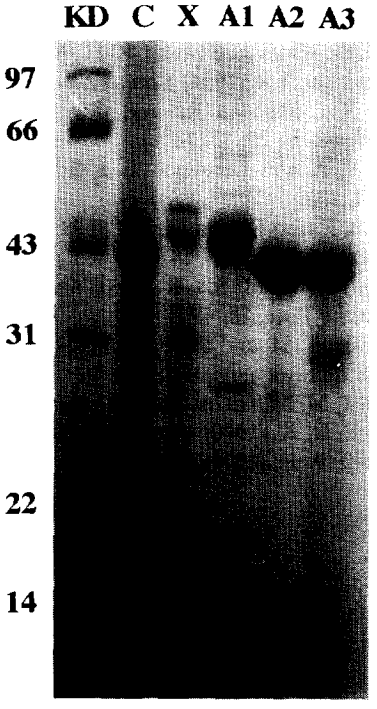

Fig. 3. SDS–polyacrylamide gel electrophoresis. Crude extracts (C, *ca* 30 μg protein), fraction X (X, *ca* 15 μg protein), and three POD isoenzymes A-1, A-2, and A-3 (15 μg protein each) purified from DEAE-cellulose shown in Fig. 2 were applied to the gel which was subsequently stained with the Coomassie Brilliant Blue R-250.

The optimum pH was 7.5 for isoenzyme A-1, 7.1 for isoenzymes A-2 and A-3, whereas the optimum temp was 37° for the three isoenzymes (Table 2). These isoenzymes had a broad pH stability between 6.5 and 11 after preincubation at 25° for 14 hr in each buffer. The three isoenzymes retained over 80% of their activity after preincubation for 10 min at 50°, indicating a high level of thermal stability (data not shown).

The three isoenzymes exhibited typical Michaelis–Menten type saturation curve for pyrogallol and H_2O_2 as determined by Lineweaver–Burk plots (data not shown). The K_m values (mM) of isoenzymes A-1, A-2, and A-3 for pyrogallol were 5.70, 2.53, and 7.14, respectively, whereas those of isoenzymes A-1, A-2 and A-3 for H_2O_2 were 2.65, 5.75, 2.78, respectively (Table 2).

Little work has been done on the POD of sweet potato (*I. batatas*) compared with other major crops. Floris *et al.* [19] have purified a neutral POD (p*I* 7.3) in a very low yield (0.9 mg from 34 g of total crude protein) as a major POD from seedlings of sweet potato. Neves and Lourenco [19] reported that the crude POD extracts derived from roots of sweet potato showed maximum activity at pH 6.5, showing a slight difference from our results (pH 7.1–7.5). Kokubu and Nakakawaji [20, 21] investigated the variations of POD isoenzymes in semituberous and fiberous roots of sweet potato varieties and its wild relative species in respect to their use in breeding. They observed six bands on the cathodic side and 22 on the anodic side. The major POD isoenzyme A-2 isolated in this study was many times higher than in intact tissues of sweet potato [17].

EXPERIMENTAL

Plant material and cell culture. The cell line SP-47 selected by a small cell-aggregate method from cell suspension cultures of sweet potato [*Ipomoea batatas* (L.) Lam. cv White Star] for a high yield of POD was used [17]. One gram (fr. wt) of cells subcultured at 10-day intervals were inoculated into 50 ml of LS (Linsmaier and Skoog [22]) basal medium supplemented with 1 mg^{-1} 2,4-D and 30 g^{-1} sucrose in a 300 ml conical flask at 25° in the dark (100 rpm).

Enzyme purification. All procedures were carried out at 4°. 226 g (fr. wt) of 21-day cultured-cells was homogenized with 10 mM KPi buffer (pH 6, 1:10, w/v) using a homogenizer with glass beads. The homogenate was centrifuged at 12000 *g* for 20 min. The resulting supernatant was concd to 100 ml by a Holow Fiber Concentrator (Amicon, M_r cut-off: 10000) which is designated as the crude extract. The crude enzyme extract was loaded onto a DEAE-cellulose (12 × 2.2 cm) previously equilibrated with extraction buffer. The enzyme was eluted with a linear gradient of 0–500 mM NaCl (100 ml each by a Pharmacia GM-1 mixer) after eluting the proteins of non-absorbing to the column. The DEAE active frac-

tions were combined and concd to *ca* 5 ml by ultrafiltration (Amicon YM 10 filter), which was further purified by Sephadex G-100 (82 × 1.6 cm).

Enzyme characterization. The effect of pH on the enzyme activity was determined using 50 mM NaOAc buffer, 50 mM K-Pi buffer and 50 mM Na borate buffer depending upon the pH range studied. The pH stability of the enzyme activity was measured after preincubation at 25° for 14 hr in each buffer. The effect of temp. on POD activity was determined at 20–80°. The temp. stability was measured after preincubation for 10 min in 100 mM K-Pi buffer (pH 6) at each temp., ranging from 20 to 80°. The K_m value for pyrogallol at a constant concn of 0.147 M H_2O_2 was obtained with the Lineweaver–Burk plot, whereas that of H_2O_2 was obtained at a constant concn of 0.4 M pyrogallol. TSK gel G3000SW (Toso, Tokyo) HPLC column (60 × 0.75 cm) was used for M_r estimation.

Enzyme assay and protein determination. The POD activity was determined by the Sigma method using pyrogallol as a substrate. The standard assay reaction mixture contained, in a total vol. of 3 ml: enzyme soln (0.1 ml), 100 mM K-Pi buffer (pH 6, 0.32 ml), 5% pyrogallol (0.32 ml, w/v), 0.147 M H_2O_2 (0.16 ml) and H_2O (2.1 ml). The reaction was initiated by the addition of H_2O_2 and the increase in $A_{420\,nm}$ recorded in 20 sec. One unit of POD activity is defined as that forming 1 mg of purpurogallin from pyrogallol in 20 sec at pH 6 at 20°. Protein was determined according to the method of ref. [23] using Bio-Rad protein assay reagents.

Electrophoresis. SDS–polyacrylamide gel electrophoresis was conducted according to ref. [24] using 12.5% gel. Bio-Rad low molecular standards were used and gels were stained with the Coomassie Brilliant Blue R-250. Isoelectric focusing was performed using a modified method of ref. [25].

Acknowledgements—This work was supported by Grants-in-Aid (E71830) from the Korean Ministry of Science and Technology. We thank Dr Seung-Ho Kim for his critical reading of this manuscript.

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