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Short communication

Peroxidase activity in calluses and cell suspension cultures of radish *Raphanus sativus* var. Cherry Bell

OSCAR A. MORENO V., RAFAEL VAZQUEZ-DUHALT* & JOSE LUIS OCHOA

*Centro de Investigaciones Biológicas de Baja California Sur, Apartado Postal 128, La Paz B.C.S., 23000 México (*requests for offprints)*

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Abstract. Calluses of *Raphanus sativus* var. Cherry Bell were induced in a medium containing 2,4-dichlorophenoxyacetic acid and benzyladenine. The biomass and peroxidase activities were determined, both in agar cultures and cell suspension cultures. Different growth-regulator concentrations induced different responses measured as peroxidase activity in callus. The suspended-cell cultures showed the importance of selecting the cell line, in order to obtain an optimal response in extracellular peroxidase activity. The commercial production of this enzyme by utilizing plant cell tissue cultures is discussed.

Introduction

Recently, the possibility of commercial production of horse-radish peroxidase by plant cell tissue culture technique has been discussed [1]. So far, the only source of commercial peroxidase (E.C. 1.11.1.7) available is from horse-radish roots, which normally are cultivated in countries with temperate climates, although it has also been found in a wide range of plants and micro-organisms [2]. The enzyme peroxidase is used in analytical biochemistry for determination of H_2O_2 in biological systems and for the labelling of antibodies and antigens [3]. New applications for peroxidase have been suggested, e.g., elimination of phenolic and aromatic compounds in wastewater treatment [4, 5].

The present work shows that cell culture of *Raphanus sativus* var. Cherry Bell in suspension could be considered another source of peroxidase enzyme.

Materials and methods

Calluses were induced from the stem, leaf and root tissues of radish plantlets

on a Murashige & Skoog basal agar medium [6] containing 2,4-dichlorophenoxyacetic acid (2,4-D) and/or benzyladenine (BA) in different proportions. The concentrations of 2,4-D and BA used were 0, 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} M. Root tissues were surface-sterilized with NaClO (5.3%) for 10 min and washed three times with sterilized distilled water. Stem and leaf tissues were obtained from plantlets produced under aseptic conditions in Murashige & Skoog basal agar medium from seeds treated with NaClO as were the roots. Seed germination was carried out at 25 °C. After a seven-week culture period at 25 °C under continuous light, one portion of the calluses were weighed and dried at 70 °C during 24 h, cooled and weighed again to determine dry weight; the other part was transferred into Erlenmeyer flasks containing 100 ml of Murashige & Skoog liquid basal medium containing the growth-regulator concentrations on which the callus was grown. The cells in suspension culture were grown at 25 °C and shaken at 150 rpm.

Protein concentration and peroxidase activity were assayed as follows. Callus samples were homogenized in a mortar with 2 ml of 0.06 M phosphate buffer (pH 6.1) at 4 °C, and centrifuged at 6700 *g*. The supernatant was assayed for protein by the method of Lowry [7] and the peroxidase activity was estimated from the increase in absorbance at 470 nm in 3 ml of phosphate buffer containing 0.016 M of guaiacol and 0.002 M of hydrogen peroxide at 25 °C according to Castillo et al. [8]. The determinations were done in triplicate.

Results and discussion

Callus induction from root, stem and/or leaf of *Raphanus sativus* var. Cherry Bell was obtained with several combinations of BA and 2,4-D concentrations in agar medium. For the three tissue types, the culture medium containing 10^{-7} M BA and 10^{-6} M 2,4-D was found satisfactory for biomass production (Table 1).

Callus obtained from *Raphanus sativus* showed different peroxidase specific activities as shown in Table 1. The heterogenous response in peroxidase activity has been reported for horse-radish callus [1]. The maximal peroxidase activities in agar cultures (in $\Delta A_{470}\,min^{-1}\,mg\,prot^{-1}$) were 305.3 in the callus from stem, 235.5 in the root callus and only 150.1 in the callus from leaf tissues.

For the liquid media, the specific activity of the peroxidase from the cell biomass and in extracellular medium after 5 weeks in the 5 selected growth-regulator combinations are presented in Table 2. Each cell suspension

Table 1. Biomass production and peroxidase specific acitivity of the five most favourable growth-regulator combinations for callus induction of different tissues of *Raphanus sativus*.[1]

Growth regulator concentration (M)		Callus weight (mg)		Peroxidase specific activity ($\Delta A_{470}\,min^{-1}\,mg\,prot^{-1}$)
BA	2, 4-D	fresh wt	dry wt	
Root[2]				
10^{-7}	10^{-6}	405.0	48.6	140.1
10^{-7}	10^{-8}	260.0	44.2	158.6
10^{-8}	10^{-5}	330.0	39.6	104.8
10^{-6}	10^{-8}	50.0	4.5	232.5
10^{-8}	10^{-7}	n.d.[4]	n.d.	127.6
Stem[3]				
10^{-5}	10^{-6}	550.1	39.7	155.7
10^{-8}	10^{-6}	194.2	24.6	66.7
10^{-7}	10^{-6}	241.7	24.2	78.8
10^{-5}	10^{-8}	151.6	18.1	114.7
10^{-7}	10^{-7}	97.4	12.7	305.3
Leaf[2]				
10^{-5}	0	1167.2	59.2	n.d.
0	10^{-7}	809.1	55.5	n.d.
10^{-7}	10^{-6}	1051.9	54.8	111.6
10^{-8}	10^{-6}	406.6	26.9	142.6
10^{-5}	10^{-6}	451.7	22.8	150.1

[1] Data selected from one series of 25 independent cultures with different growth-regulator concentrations in each tissue type.
[2] After 7 weeks of culture
[3] After 10 weeks of culture
[4] Not determined

culture proceeded from one callus obtained in agar culture with the same growth-regulator concentration and were obtained in three independant replicates. Biotechnologically, the extracellular peroxidase is more attractive, because its recovery would be simplified and its production could be achieved from immobilized cell cultures. For this reason, we have selected the culture with higher extracellular production of peroxidase activity. The highest peroxidase activity in the cell-free medium was found with 10^{-7} M BA and 10^{-6} M 2,4-D concentration. There was no correlation between specific activity of the peroxidase in the callus and in the cell suspension cultures.

The kinetics of the production of extracellular protein and extracellular peroxidase activity in cell suspension culture was determined in cultures

Table 2. Peroxidase specific activity in cell biomass and free medium in cell suspension culture from root callus of *Raphanus sativus* var. Cherry Bell, after five weeks of culture.

Growth regulator concentration (M)		Peroxidase specific activity ($\Delta A_{470}\,min^{-1}\,mg\,prot^{-1}$)	
BA	2,4-D	in cell biomass	in cell-free medium
10^{-7}	10^{-6}	65.3 (± 9.7)[1]	84.0 (± 12.7)
10^{-6}	10^{-7}	45.2 (± 24.0)	34.3 (± 15.4)
10^{-6}	10^{-6}	108.0 (± 18.1)	34.5 (± 4.4)
10^{-6}	10^{-5}	17.0 (± 0.1)	34.4 (± 0.2)
10^{-5}	10^{-7}	97.5 (± 0.4)	68.8 (± 3.0)

[1] The standard deviation (in parentheses) was calculated from three values obtained in independent replicate experiments.

grown in 10^{-7} M BA and 10^{-6} M 2,4-D cell suspension culture. One callus tissue from agar medium with 10^{-7} M BA and 10^{-6} M 2,4-D was put into liquid medium with the growth-regulator concentrations on which callus was grown and cultured during 4 weeks. Then, 10 ml of the cell suspension culture were taken and inoculated in 5 parallel cultures. As shown in Figure 1A, the extracellular protein of *R. sativus* was about the same in all experiments, reaching values near 300 $\mu g\,ml^{-1}$ after the first week. Prolonged culturing yielded a similar amount of extracellular protein even after 8 weeks. In contrast, we have found two kinds of responses for peroxidase activity: one in which the enzyme in extracellular medium shows low constant levels and another, where the activity increases during a three-week period of culture, decreasing subsequently to lower values (Fig. 1B). In all cases the enzyme activity was preceded by extracellular protein production (Fig. 1A and 1B).

Extracellular peroxidase has been reported in suspension cultures of carrot [9], peanut [10], potatoes [11], spinach [12], cotton [13] and horseradish [1]. In the case of petunia, the peroxidase secreted into the extracellular medium can reach 15% of the total secreted protein [10]. Different responses with respect to peroxidase activity in calluses of horseradish cultures has recently been observed [1]. Therefore, cell line selection was suggested in order to obtain a cell line with high peroxidase activity in cell tissue cultures [1]. In our case, using cell suspension cultures from one cell aggregate, it appears that the selection of cell lines with high peroxidase production can be carried out directly from liquid culture.

The reduction of peroxidase activity in the extracellular medium after the third week of culture (Fig. 1B) can be attributed to exhaustion of available calcium in the medium. Reactivation of extracellular peroxidase from the

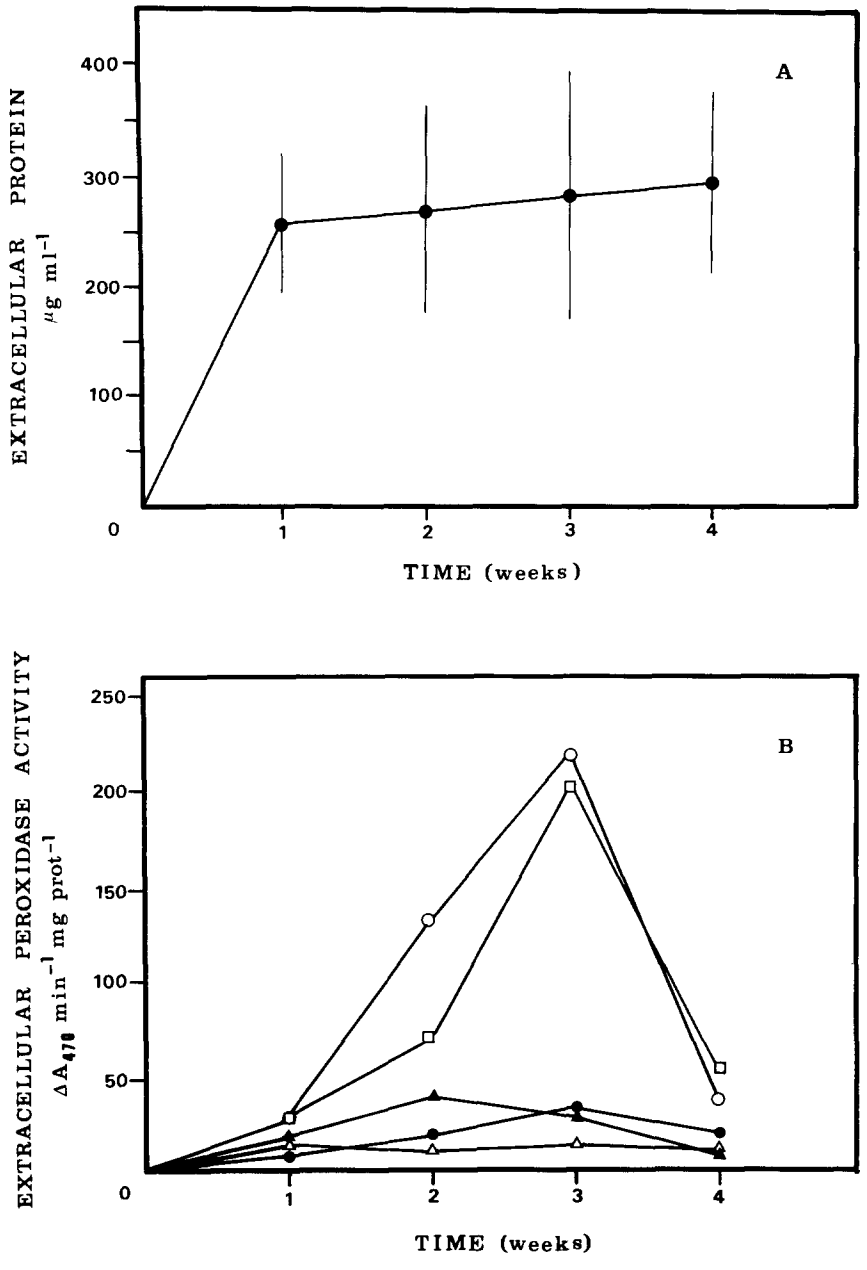

Fig. 1. Protein and peroxidase activity in the medium during cell suspension culture of *Raphanus sativus*. (*A*) Average values of extracellular protein from five experiments. (*B*) Peroxidase activity of cell-free medium; each symbol represents one parallel experiment. The medium with 10^{-7}M BA and 10^{-6}M 2,4-D, containing cell biomass from root tissue, was cultured as described in 'Materials and methods'.

Table 3. Reactivation of peroxidase activity from extracellular medium after four weeks of cell suspension cultures.

Experiment	Peroxidase specific activity ($\Delta A_{470}\,min^{-1}\,mg\,prot^{-1}$)	
	no $CaCl_2$ added	5 mM $CaCl_2$ added
I (□)[1]	53.12	34.75
II (●)	20.03	69.50
III (○)	38.39	721.73
IV (△)	11.56	48.12
V (▲)	8.58	45.29

[1] The symbols in parenthesis represent the corresponding data shown in Figure 1B.

fourth week of suspension cultures by calcium is showed in Table 3. In many instances, calcium activates plant peroxidases and its absence inactivates peroxidase [8, 14, 15]. It is a well-documented fact that suspension-cultured cells secrete macromolecules [16]. These secreted molecules could chelate the calcium.

Noteworthy, the specific activity of purified commercial horse-radish peroxidase ($1380\,\Delta A_{470}\,min^{-1}\,mg\,prot^{-1}$) is only four times that of crude extract of cell aggregates of *Raphanus sativus* var. Cherry Bell ($305.3\,\Delta A_{470}\,min^{-1}\,mg\,prot^{-1}$). Thus it may be possible to purity the *Raphanus sativus* peroxidase to a higher specific activity than the horse-radish enzyme.

In conclusion, these results show that the peroxidase production from cell-suspended cultures of *Raphanus sativus* var. Cherry Bell should be considered as a commercial alternative to horse-radish roots.

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