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Effect of Chitosan on Peroxidase Activity and Isoenzyme Profile in Hairy Root Cultures of *Armoracia lapathifolia*

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Abstract

Hairy root cultures of *Armoracia lapathifolia* established by infection with *Agrobacterium rhizogenes* LBA 9402 present a level and isoenzyme pattern of peroxidases (POD) comparable to nontransformed roots. Elicitation with chitosan (10, 50, and 100 mg/L) was used in order to improve POD production. Total POD activity increased about 170% after 48 h of treatment with chitosan 100 mg/L. Elicitation effect on soluble and ionically cell-wall-bound POD fractions of *A. lapathifolia* hairy roots was analyzed. POD activity of the ionically cell-wall-bound protein fraction increased in the presence of chitosan in a dose–response manner. No effect on soluble POD fractions was observed, but the isoenzyme pattern analyzed by isoelectrofocusing showed an increase of an acidic isoenzyme (p*I* = 3.4) after the elicitation treatment. The ionically cell-wall-bound protein fraction showed only basic isoenzymes, with an increase of an isoenzyme of p*I* = 8.7, after the elicitation treatment.

Index Entries: *Armoracia lapathifolia*; transformed root culture; peroxidase production; chitosan; elicitation.

Introduction

Peroxidases (POD, E.C. 1.11.1.7) are ubiquitous hemoproteins that catalyze the oxidation of a wide variety of substrates by using hydrogen peroxide. The broad substrate specificity and polyfunctionality and availability of peroxidases from different sources permit the application of these enzymes to various biotechnological processes.

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Plant peroxidases have wide commercial applications. They can be applied both in industrial processes and as analytical tools. In industrial processes, peroxidases have been used to produce food colorants, dyes for perfume industries, biobleaching *(1)*, and removal of toxic and carcinogenic pollutants from industrial effluents *(2–5)*.

The basic peroxidases are used as reagents for diagnostic test kits *(6)* and markers in inmunoassay *(7)* and in the preparation of DNA probes *(8)*.

Horseradish roots are the most common commercial source of peroxidase, occurring as multiple isoenzymes *(9)*. Because horseradish production from field-grown plants may be affected by reproductive and environmental factors, there is considerable interest in exploring in vitro plant cultures *(10)* and, particularly, transformed root cultures *(11,12)*. These roots, obtained by transformation with *Agrobacterium rhizogenes*, have specific advantages like genetic and biochemical stability, fast growth in hormone-free media under defined aseptic conditions with low cost of implementation, and easy maintenance. These properties deserve their use in biotechnological processes.

In a previous work *(13)*, we reported the production of peroxidases by hairy root cultures of *Armoracia lapathifolia*, which present an enzymatic activity level and isoenzymes profile like roots from field-grown plants. When the cultures were elicited by fungal homogenates, peroxidase activity was increased about 100%.

As is well known, elicitation is a common strategy for increasing yields of secondary metabolites in *in vitro* plant cell cultures *(14,15)*. For that purpose, fungal homogenates of different species have been recommended. The results depend on the fungal species employed and also on the homogenate preparation *(16,17)*.

In order to obtain a reproducible result, the use of elicitors of chemically defined composition such as chitosan is advisable *(18)*. Chitosan (polycationic polymer of β-1-4-linked D-glucosamine) is the major component of exoskeletons of insects and Crustacea and can be found in the cell walls of many fungi.

The aim of this work is to study the effect of chitosan on peroxidase level and isoenzyme profile in hairy root cultures of *A. lapathifolia*.

Material and Methods

Hairy Root Culture Initiation and Maintenance

Hairy roots were induced by infection with *A. rhizogenes* LBA 9402 as described previously *(13)*. Transformation was confirmed by polymerase chain reaction (PCR). The hairy root cultures were maintained in hormone-free Murashige and Skoog liquid medium *(19)*, containing the vitamin complex of the revised tobacco medium *(20)* and supplemented with 3.0% (w/v) of sucrose (this medium will be referred to as MSRT hereafter). Incubation was carried out on a gyratory shaker at 100 rpm, at 24 ± 2°C, under a 16-h photoperiod by using cool white fluorescent lamps at a light

intensity of approx 1.8 W/m^2. The hairy roots were subcultured every month, in the same medium and culture conditions described.

Chemicals

All of the media components, acetic acid, and chitosan (from crab shells, with degree of deacetylation of 72%) were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Elicitation Assay

Elicitation was carried out with chitosan. A stock solution of 20 g/L was prepared by dissolving chitosan in 1% acetic acid. A viscous solution was obtained after agitation overnight. Then, it was autoclaved at 121°C for 20 min and the sterile solution was employed for the elicitation assay.

For elicitation assays, approx 100 mg fresh weight (FW) of 30–d-old *A. lapathifolia* hairy root tips were transferred to 125-mL Erlenmeyer flasks containing 20 mL of MSRT medium and cultured as previously described. After this time, aliquots of the stock chitosan solution were added up to reach a final concentration of 10, 50, and 100 mg/L in the culture media. The two controls used were prepared by adding a volume of sterile distilled water or 1% acetic acid instead of chitosan. The acetic acid volume corresponds to the highest concentration of the elicitor agent. In each case, the hairy roots were exposed for periods of 24 and 48 h under culture conditions previously described.

After each treatment, POD activity was estimated in whole hairy roots, in soluble and ionically cell-wall-bound fractions of hairy roots, and also in culture media. In addition, the isoenzyme pattern was determined in soluble and ionically cell-wall-bound fractions.

Preparation of Enzyme Fractions

To extract the soluble PODs, a sample of 50 mg of hairy roots was homogenized in a mortar with 1.5 mL of 50.0 m*M* phosphate buffer (pH 6.0). The homogenate was centrifuged at 16,000*g* for 10 min and the resulting supernatant kept for determination of the soluble POD activity. The pellet was washed repeatedly with the same buffer until no POD activity was found. Ionically, cell-wall-bound PODs were released by mixing the washed pellet with 1 mL of 50 m*M* phosphate buffer–1.0 *M* KCl (pH 6.00) and incubated overnight at 18–20°C. Then, the homogenate was centrifuged for 10 min at 13,000 rpm and the supernatant saved for determination of ionically cell-wall-bound POD activity.

Enzyme Assay

Peroxidase activity was measured in the soluble and ionically cell-wall-bound fractions of hairy roots. Peroxidase activity was determined following the oxidation of guaiacol at 470 nm, as previously described *(13)*.

Briefly, it employed guaiacol as the electron donor (30 m*M* in sodium phosphate buffer, pH 7.4) in the presence of H_2O_2 (10 m*M*) as the electron acceptor, plus 10 µL of the corresponding enzyme extract. The final reaction volume was 3 mL. One enzymatic unit (U) was arbitrarily defined as the amount of enzyme that produces a change in A_{470} of 0.021 per minute at 25°C under previously defined conditions.

Isoelectric Focusing and In Situ *POD Activity Staining*

Tissue extracts corresponding to soluble and ionically cell-wall-bound POD fractions were subjected to isoenzyme analysis. Hairy root homogenates were previously desalted with Sephadex G-25. Their isoenzyme patterns were analyzed by isoelectric focusing (IEF) in a polyacrylamide gel with a pH range of 3–10 using a Bio-Rad Mini IEF system according to the manufacturer's instructions. The ampholyte concentration was reduced to 1% (w/v) in order to obtain a better consistency of the gels. To detect POD activity, the gels were stained with freshly prepared *o*-dianisidine reagent (0.25 m*M* in 50.0 m*M* phosphate buffer, pH 6.0). The reaction was started by adding 50.0 µL of H_2O_2 in 50.0 mL of the *o*-dianisidine solution. The color was developed under constant agitation for 15 min. In order to stop the reaction, the gel was washed with distilled water.

Statistical Analysis

One-way analysis of variance (ANOVA) and Tukey's honestly significant difference (Tukey's HSD) tests were performed to compare treatment means (confidence level of 95%).

Results and Discussion

Effect of Elicitation Treatment With Chitosan on Peroxidase Activity

Figure 1A shows the effect of chitosan on POD activity in 14-d-old *A. lapathifolia* hairy root cultures. Elicitation treatments with chitosan were applied to 14-d-old cultures (at the end of the exponential growth phase). In previous work *(13)*, we found that peroxidase levels of hairy root cultures of *A. lapathifolia* began to rise at this stage of culture. Total POD activity registered an increase of about 170% after 48 h of treatment with chitosan concentrations of 100 mg/L. This increment was higher than those obtained by elicitation of *A. lapathifolia* hairy roots with fungal extracts *(13)*. Because chitosan was added to the cultures in a solution of acetic acid, it was necessary to check the effect of this reagent alone. Irrespective of the POD fraction analyzed, we found that acetic acid produced an increase in POD activity comparable to that produced by 10 mg/L of chitosan. No toxic effects of acetic acid on the roots were observed during the time assayed. The effect of acetic acid could be related to a possible destabilization of the membrane, producing changes in ion fluxes, which would further induce POD activity *(21)*.

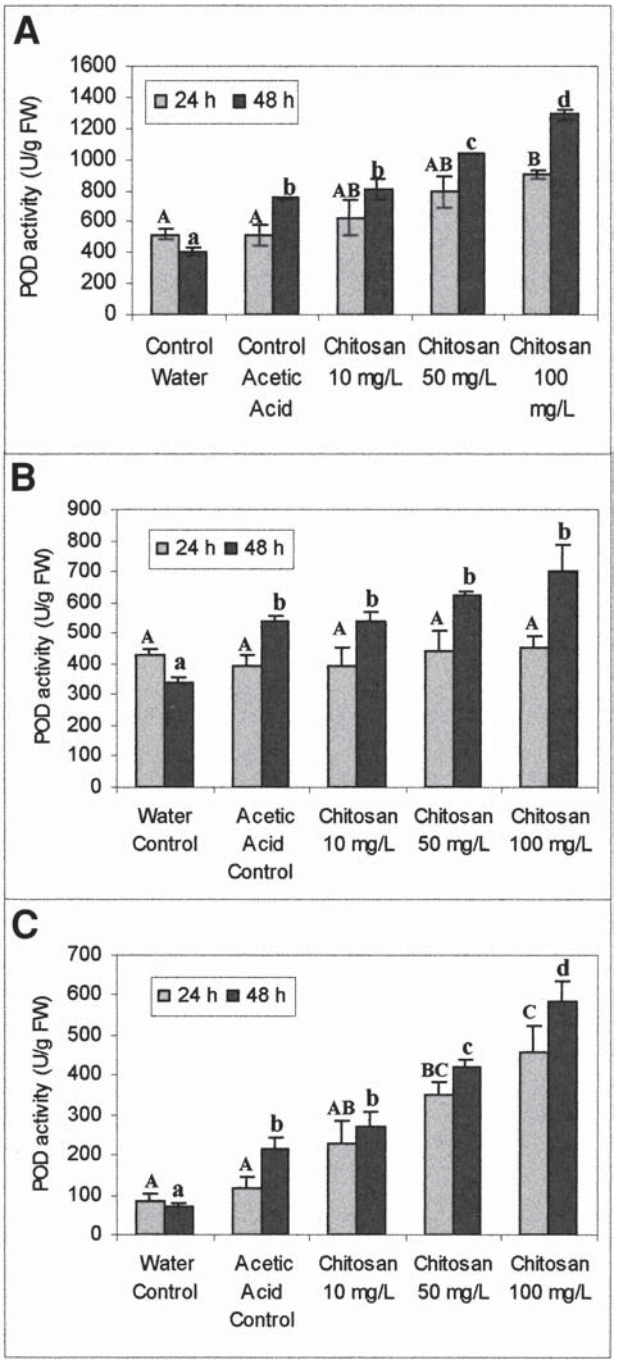

Fig. 1. Effect of chitosan on POD activity of *A. lapathifolia* hairy root cultures, after 24 and 48 h of treatment: **(A)** effect on the total POD activity; **(B)** effect on soluble fraction; **(C)** effect on ionically cell-wall-bound fraction. The same letter indicates no statistical significant difference (Tukey's HSD; $p < 0.05$). Capital letters corresponds to the 24-h treatment series. Lowercase letters correspond to the 48-h treatment series.

Figure 1B,C show that the effect of the elicitation treatment on POD activity of transformed root cultures of *A. lapathifolia* varied depending on the fraction of enzymes analyzed. Figure 1B shows the activity of soluble POD fraction after 24 and 48 h of elicitation treatment. No effect on POD activity was observed, either after 24 or 48 h.

A different response to the elicitation treatment was obtained in the case of the ionically cell-wall-bound fraction of POD (Fig. 1C); after 24 h of treatment, a notable increase in POD activity was observed, reaching approximately a fourfold increase in the case of treatment with 100 mg/L of chitosan. After 48 h, the increase of POD activity continued and showed a profile similar to that found after 24 h of treatment: POD activity increased in a dose–response manner, being the highest level (581.7 ± 50.81 U/g FW) obtained with 100 mg/L of chitosan. A linear correlation was found between POD activity of ionically cell-wall-bound fraction and the amount of chitosan employed as the elicitor agent. The data were plotted taking the POD level corresponding to the control of acetic acid as the level of POD corresponding to 0 mg/L of chitosan (data not shown). A higher correlation coefficient was obtained for the dataset corresponding to 48 h of elicitation treatment ($R^2 = 0.9647$ for 48 h and $R^2 = 0.8641$ for 24 h). The slope of both curves is similar and the initial POD activity value is higher for the set corresponding to 48 h of treatment, showing that the effect of acetic acid is more marked after 48 h of treatment. No POD activity was detected in the culture media in any case.

When the soluble and ionically cell-wall-bound fraction of PODs were analyzed, a differential response to elicitation was found. Soluble fractions did not respond to the treatment with chitosan. Ionically cell-wall-bound POD levels increased markedly as a consequence of the elicitation treatment. This phenomenon could be related to the possible role of cell-wall-bound POD isoenzymes. They are considered as the main candidates to catalyzed reactions involved in defense mechanisms *(22,23)*. For instance, it has been shown that fungal elicitors in cell suspensions of spruce *(24)* induce this fraction. Also, the ionically bound fraction increased significantly in bean seedlings inoculated with pathogenic *Rhizoctonia* spp. *(25)*. These data suggest that cell-wall fractions might have a role in secondary wall biosynthesis and lignification, both mechanisms possibly directed to protect against the invasion of pathogens. Chitosan possesses a oligosaccharide structure present in many fungal cell walls and it is known to trigger plant defense responses, including the production of phytoalexins and structural components, protease inhibitors, and pathogenesis-related proteins like chitinases and PODs *(26)*.

Effect of Elicitation on Isoenzyme Pattern

To analyze which POD isoenzymes were affected by the elicitation treatment, samples were analyzed by IEF. We analyzed samples corresponding to the treatment with 100.0 mg/L of chitosan after 48 h. These samples were selected because the effect of elicitation could be clearly

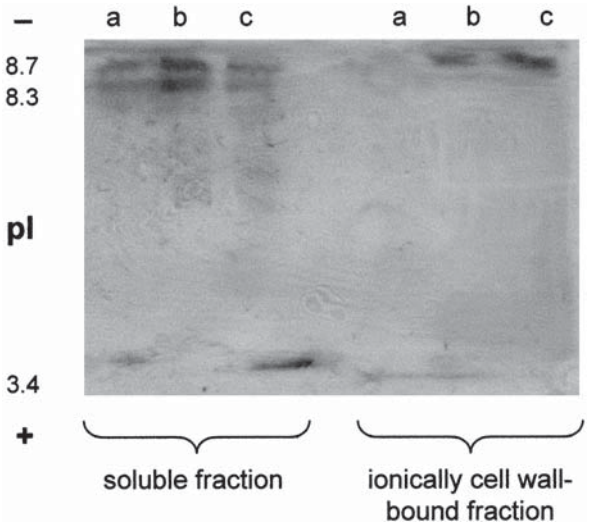

Fig. 2. Isoelectrofocusing pattern of soluble and ionically cell-wall-bound POD isoenzymes of hairy root cultures of *A. lapathifolia* after 48 h of elicitation: (a) water control; (b) acetic acid control; (c) 100 mg/L chitosan.

observed on the levels of POD detected for each enzyme fraction. Equivalent weight samples were applied to polyacrylamide gels and stained for POD activity. Figure 2 shows the isoenzyme pattern corresponding to soluble and ionically cell-wall-bound fractions. In the case of the soluble fraction, basic and acidic isoenzymes were detected, with a marked increase in an acidic isoenzyme (p*I* approx 3.4). In the case of ionically cell-wall-bound fraction, only basic isoenzymes were detected. (The isoenzymes of this fraction coincided with one of the basic isoenzymes found in the soluble fraction of p*I* approx 8.7.) It is shown that there is an increase of an isoenzyme of p*I* approx 8.7 in the case of treatment with 100 mg/L of chitosan. The same effect was observed for the control of acetic acid. The changes in POD levels observed in this fraction might be caused by variations on the basic isoenzyme levels. However, the treatment with chitosan produced an increase in POD activity of ionically cell-wall-bound POD fraction more marked than that produced by only acetic acid alone. Consequently, a more intense isoenzyme band would be expected for chitosan treatment, but it cannot be easily observed on the gel at the first sight.

Isoelectric focusing provides a rapid means for characterizing POD isoenzymes and following their behavior under different physiological and environmental conditions. Soluble and ionically cell-wall-bound fractions displayed distinctive sets of POD isoenzymes. Acidic and basic isoenzymes were found in the soluble fraction. In the case of the ionically cell-wall-bound fraction, only basic isoenzymes were found. A similar distribution of POD isoenzymes was found in horseradish tumor and teratoma tissue *(27)*. An acidic isoenzyme, which was found only in the soluble fraction (p*I* aprox 3.4) showed an increase after elicitation treatment as well as a

basic isoenzyme of the cell-wall-bound fraction (p*I* approx 8.7). An increase in the intensity of a basic isoenzyme of p*I* approx 8.6 was also observed in *A. lapathifolia* hairy roots elicited with fungal extracts *(13)*. Acidic POD isoenzymes have been related to rigidification of cell walls *(28,29)*. However, basic isoenzymes are also capable of catalyzing reactions, leading to reinforcement of cell walls *(29)*. It is possible that both isoenzymes increase, after the elicitation treatment, and could be involved in reactions tending to limit pathogen invasion (e.g., stiffening of cell walls).

We can conclude that the elicitation with chitosan increases POD activities in hairy root cultures of *A. lapathifolia* and also affects isoenzymes patterns by increasing the basic ones. It is evident that the use of chitosan is particularly attractive for commercial analytical applications, taking into account that the basic isoenzymes are widely employed for such purpose specifically for diagnostic test kits.

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References

1. Harazono, K., Kondo, R., and Sakai, K. (1996), *Appl. Environ. Microbiol*. **62,** 913–917.
2. Roper, J. C., Dec, J., and Bollag, J. M. (1996), *J. Environ. Qual*. **25,** 1242–1247.
3. Dec, J. and Bollag, J. M. (1994), *Biotechnol. Bioeng*. **44,** 1132–1139.
4. Wagner, M. and Nicell, J. A. (2001), *Water Sci. Technol*. **43,** 253–260.
5. Bhunia, A., Durani, S., and Wangikar, P. (2001), *Biotechnol. Bioeng*. **72,** 562–567.
6. Krel, H. W. (1991) in *Biochemical, Molecular, and Physiological Aspect of Plant Peroxidases*, Labarzewki, J., Greppin, H., Pelel, C., and Gaspar, T. (eds.), Imprimerie National, Geneva, pp. 470–478.
7. Tijssen, P. (1985), in *Laboratory Techniques in Biochemistry and Molecular Biology, Vol. 15*, Burdon, R. and Van Knippenberg, P. (eds.), Elsevier, Amsterdam, pp. 173–219.
8. Cercek, B., Roby, K., and Siaw, M. (1995), *Anal. Biochem*. **232,** 143–144.
9. Shanon, L. M. and Kay, J. Y. (1996), *J. Biol. Chem*. **241,** 2166–2172.
10. Yamada, Y., Kobayashi, S., Watanabe, K., Hayashi, U., Yajima, Y., and Inove, H. (1987), *J. Chem. Tech. Biotechnol*. **38,** 31–39.
11. Toivonen, L. (1993), *Biotechnol. Prog*. **9,** 12–20.
12. Kim, Y. H. and Yoo, Y. J. (1996), *Enzyme Microb. Technol*. **18,** 531–535.
13. Flocco, C. G., Alvarez, M. A., and Giulietti, A. M. (1998), *Biotechnol. Appl. Biochem*. **28,** 33–38.
14. Bohlmann, J. and Eilert, U. (1994), *Plant Cell Tissue Org. Cult*ure **38,** 189–198.
15. Pitta Alvarez, S. I., Spollansky, T. C., and Giulietti, A. M. (2000), *Enzyme Microb. Technol*. **26,** 252–258.
16. Sim, S. J., Chang, H. N., Liu, J. R., and Jung, K. H. (1994), *J. Ferm. Bioeng*. **78,** 229–234.
17. Buitelaar, R. M., Cesario, M. T., and Tramper, J. (1992), *Enzyme Microbiol. Technol*. **14,** 2–10.
18. Pitta Alvarez, S. and Giulietti, A. M. (1999), *Plant Cell Tissue Org. Culture* **59,** 31–38.
19. Murashige, T. and Skoog, F. (1962), *Physiol. Plant* **15,** 473–497.
20. Khanna, P. and Staba, J. (1968), *Lloydia* **31,** 180–189.
21. Gaspar, T., Penel, C., Castillo, F. J., and Greppin, H. (1985), *Physiol. Plant* **64,** 418–423.

22. Lamport, D. T. A. (1986), in *Molecular and Physiological Aspects of Plant Peroxidases* Grippen, H., Penel, C., and Gaspar, T. (eds.), University of Geneva Press, Geneva, pp. 199–207.
23. Lagrimini, L. M. and Rothstein, S. (1987), *Plant Physiol*. **84,** 438–442.
24. Mensen, R., Achim, H., and Salzer, P. (1998), *Physiol. Plant* **102,** 539–546.
25. Xue, L., Charest, P. M., and Jabaji-Hare, S. H. (1998), *Biolog. Control*. **4,** 359–365.
26. Benhamou, N. (1996), *Trends Plant Sci*. **1,** 233–240.
27. Peskan, T., Pedreño, M. A., Krsnik-Rasol, M., and Muñoz, R. (1997) *Biol. Plant*. **39,** 575–582.
28. Zheng, X. and Van Huystee, R. B. (1991), *Plant Cell Tissues Org. Culture* **25,** 35–43.
29. Ferrer, M. A., Pedreño, M. A., Ros Barceló, A., and Muñoz, R. (1992), *J. Plant Physiol*. **139,** 611–616.