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Phytochemistry 65 (2004) 2471–2476
PHYTOCHEMISTRY
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Alterations in intracellular and extracellular activities of antioxidant enzymes during suspension culture of sweetpotato

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Received 22 January 2004; received in revised form 10 May 2004

Abstract

Cultured plant cells are a good system for the study of antioxidant mechanisms and for the mass production of antioxidants, because they can be grown under conditions of high oxidative stress. Alterations in the intracellular and extracellular activities of three antioxidant enzymes, superoxide dismutase (SOD), guaiacol-type peroxidase (POD), and glutathione peroxidase (GPX), were investigated in suspension cultures of sweetpotato (*Ipomoea batatas*) during cell growth. Intracellular SOD activities (units/mg protein) at 15 days after subculture (DAS) and 30 DAS were 10 and 20 times higher, respectively, compared with the SOD activity at 1 DAS, whereas intracellular specific POD and GPX activities did not significantly increase until after 15 DAS, when they rapidly increased. The extracellular activities of the three enzymes in culture medium were much higher than were the intracellular activities. The change in extracellular SOD activity was similar to that of extracellular GPX during cell growth. Those activities showed high levels until 5 DAS and then significantly decreased. Extracellular POD activity had an almost constant level regardless of the cell growth stage. In addition, intracellular SOD and POD isozymes were quite different from those isozymes in the culture medium. The changes in SOD and POD isozymes observed here suggest that different isozymes might modulate the levels of reactive oxygen intermediates during cell growth. Characterization of extracellular antioxidant enzymes discovered here would provide a new understanding for defense mechanism in plants.

Keywords: Sweetpotato *Ipomoea batatas*; Convolvulaceae; Suspension cultures; Cell growth; Intracellular and extracellular antioxidants; Isozyme analysis; SOD; POD; GPX

1. Introduction

Reactive oxygen intermediates (ROIs), such as either superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2), or hydroxyl radicals (OH^-) oxygen $(O_2)'$ are produced as by-products of normal cell metabolism, although environmental stresses can cause an increase in their levels. ROI levels must be controlled, because an over accumulation of ROIs can result in cell death (Asada and Takahashi, 1987; Asada, 1999; Dat, 2000). ROI-induced cell death can result from oxidative processes such as membrane lipid peroxidation, protein oxidation, inactivation of enzymes, alteration of intracellular redox state, and damage to DNA. ROIs are thought to participate in the pathogenesis of a number of diseases and in the aging process (Mittler, 2002). Plants have defense systems that protect them against toxic ROIs. The resistance of a plant to stress is correlated with its increased

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doi:10.1016/j.phytochem.2004.08.001

capacity to scavenge or detoxify ROIs. The best well known antioxidant enzymes are intracellular enzymes such as superoxide dismutase (SOD), peroxidase (POD), glutathione peroxidase (GPX), catalase (CAT), and ascorbate peroxidase (APX), which protect against the toxic effects of oxidants generated within cells.

Recently, SOD and GPX were characterized in the cell culture medium and the extracellular space of mammalian cells (Comhair et al., 2001; Lindmark-Månsson and Åkesson, 2001; Ookawara et al., 2003). Ookawara (2003) reported upregulated translocation of extracellular SOD (a Cu/Zn-containing secretory glycoprotein) from the medium to the nucleus in 3T3-L1 cells under oxidative stress, suggesting that SOD plays a role in protecting the nucleus against oxidative damage to genomic DNA. In human BET1A cells, extracellular GPX expression was increased in response to ROI, providing clear evidence for the redox regulation of expression (Comhair et al., 2001). There are, however, no reports on extracellular SOD and GPX in plants.

Cultured plant cells are a good system for the study of both intracellular and extracellular antioxidant enzymes, because they can be grown under conditions of high oxidative stress. Plant cell suspension cultures are very important in the field of plant biotechnology for the development of transgenic plants, the biosynthesis of key pharmaceutical proteins, and mass propagation. We previously investigated the levels of intracellular antioxidant enzymes such as SOD, POD, and CAT, and low molecular antioxidants (ascorbate and glutathione), in 100 cell lines derived from different plant species. Cultured plant cells were found to have much higher levels of antioxidant enzymes such as POD and SOD than did whole plant tissues (Kim et al., 1994, 1999; You et al., 1996). The level of catalase activity and the amount of ascorbate were lower in cultured plant cells than in whole-plant tissues (Jang et al., 1996; Ahn et al., 1998). The level of glutathione (GSH) was similar in both cultured cells and whole plants (Lee et al., 2000). Recently, Kim et al. (2003) isolated and characterized a novel oxidative-inducible POD (*SWPA2*) promoter from sweetpotato (*Ipomoea batatas*) that was highly expressed in suspension cultures. The *SWPA2* promoter is being used to develop transgenic plants with enhanced tolerance to environmental stress and transgenic cell lines for the production of key pharmaceutical proteins.

In this work, we investigated the changes in the intracellular and extracellular activities of three antioxidant enzymes, SOD, POD, and GPX, during cell growth of sweetpotato in suspension cultures. The analysis of the cell phase-dependent induction of the isozymes of these enzymes and their release into the culture medium would provide valuable information for identification and characterization the isozymes responsible for oxidative culture stress during cell growth. This is the first investigative report of extracellular SOD and GPX in plants.

2. Results and discussion

2.1. Protein levels during cell growth of sweetpotato

During 30 days in culture, sweetpotato suspension cells (*I. batatas* (L) Lam. cv. White Star) showed a typical sigmoidal growth curve with an initial lag phase of five days after subculture (DAS), followed by an exponential phase of up to 20 DAS, and then a stationary phase of up to 30 DAS (Fig. 1(a)). Cell growth was maximal at 25 DAS. Intracellular protein content (mg/g cell fresh wt) continuously decreased with cell growth (Fig. 1(b)), whereas extracellular protein content (mg/ml culture medium) increased (Fig. 1(c)).

2.2. Intracellular antioxidant enzyme activities during cell growth of sweetpotato

Alterations in the intracellular antioxidant activities of SOD, POD, and GPX were investigated at different time points during cell growth of the suspension-cultured sweetpotato. Intracellular SOD activities (units/mg protein) at 15 DAS and 30 DAS were 10 and 20 times higher, respectively (Fig. 2(a)), compared with the SOD activity at 1 DAS, whereas intracellular specific POD and GPX activities did not significantly increase until 15 DAS and then rapidly increased (Fig. 2(b) and (c)). Maximal specific activities of intracellular SOD and POD were shown at 30 DAS. However, intracellular-specific GPX activity (units/mg protein) reached a maximum at 25 DAS and then rapidly decreased.

2.3. Extracellular antioxidant enzyme activities in the culture medium during cell growth

Antioxidant enzymes have been discovered in the culture medium from several mammalian cell types, and their functions against ROIs have been investigated (Adachi et al., 2000; Ookawara, 2003; Comhair et al., 2001). In plants, only PODs from the culture medium of tobacco and peanut cells have been isolated and characterized (Buffard et al., 1990; de Marco et al., 1999). It was of interest to investigate extracellular SOD and GPX, as well as extracellular POD, in the medium of suspension cultures of sweetpotato during cell growth. The change in specific activity of extracellular SOD (units/mg protein) was similar to that of extracellular GPX during cell growth (Figs. 3(a) and 2(c)). Both showed high activity levels until 5 DAS, and then the activities significantly decreased until 10 DAS. In contrast, the specific activity of extracellular POD remained nearly constant in the medium regardless of the cell

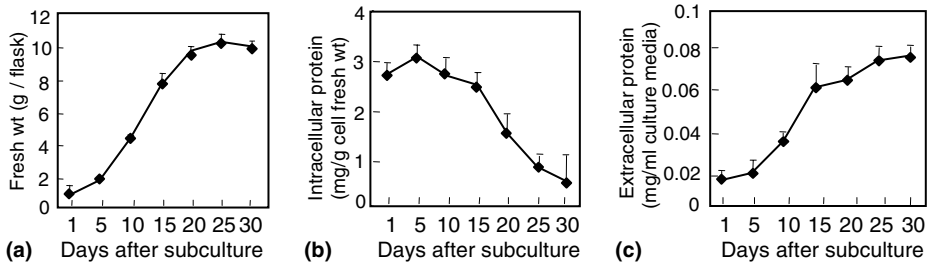

Fig. 1. Intracellular and extracellular protein levels of suspension-cultured sweetpotato in MS basal medium supplemented with 1 mg/l of 2,4-dichlorophenoxyacetic acid and 30 g/l of sucrose. (a) Time course of cell growth on the basis of cell fresh wt (g). (b) Time course of intracellular protein content (mg protein/g cell fresh wt). (c) Time course of extracellular protein content (mg protein/ml culture medium) released from the cells into the culture medium. Data are means ± SE of three independent culture flasks.

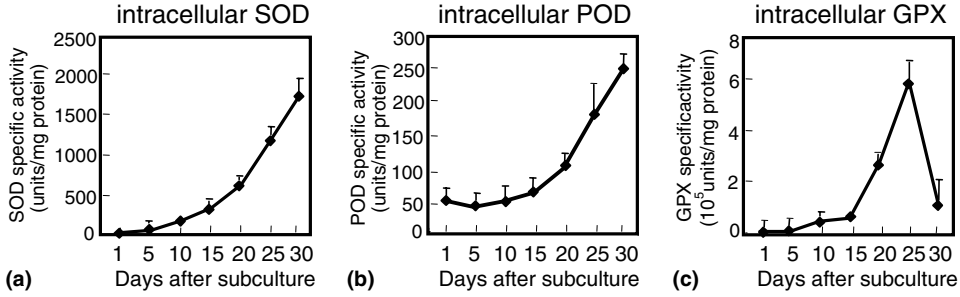

Fig. 2. Intracellular enzyme activities: (a) Specific SOD activity during cell growth. (b) Specific POD activity during cell growth. (c) Specific GPX activity during cell growth. Data are means ± SE of three independent replicas.

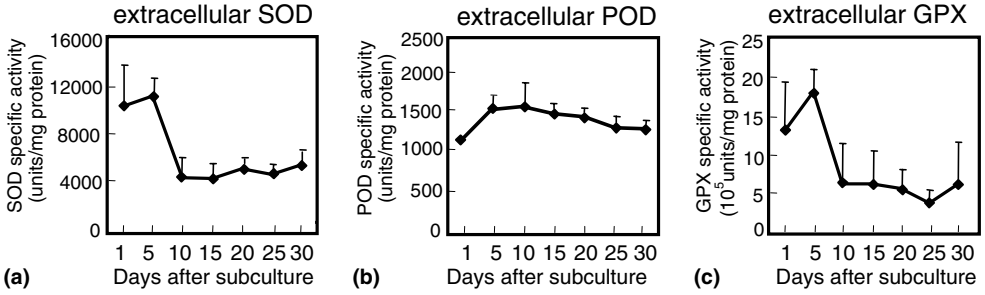

Fig. 3. Extracellular enzyme activities. (a) Specific SOD activity in culture medium during cell growth. (b) Specific POD activity in culture medium during cell growth. (c) Specific GPX activity in culture medium during cell growth. Data are means ± SE of three independent replicas.

growth stage (Fig. 3(b)). Extracellular sweetpotato POD activity differed from extracellular tobacco POD activity, which is undetectable until the cell-proliferation phase but becomes abundant during the cell-expansion phase (Narita et al., 1995; de Marco et al., 1999). The tobacco POD functions physiologically in cell wall assembly and in the control of cell wall plasticity during cell elongation. The sweetpotato suspension cultures were selected as a high yield of POD cell line by Kim et al. (1994). The high POD activity in sweetpotato suspension culture in the present study confirms again sweetpotato cells as a high yield system of POD; this result suggests that sweetpotato suspension cells maybe respond differently in generating antioxidant enzymes such as POD from tobacco cells. In addition, further characterization of extracellular SOD and GPX discovered here would provide a new direction to understand defense mechanism against oxidative stress in plants.

The extracellular specific activities (units/mg protein) of SOD, POD, and GPX, as well as the extracellular total activities (units/flask) of all three enzymes, were higher than the respective intracellular enzyme activities throughout cell growth (data not shown).

2.4. Analysis of intracellular and extracellular SOD and POD isozymes

The isozymes of SOD and POD were analyzed by native polyacrylamide gel electrophoresis (PAGE) of cells and culture medium at different points during cell

growth. The SOD isozymes were distinguished using specific enzyme inhibitors (data not shown). The bands that disappeared upon CN^- treatment represented Cu/ZnSOD, and the band that was sensitive to H_2O_2 represented a FeSOD. The band that was insensitive to both CN^- and H_2O_2 was likely MnSOD. Intracellular SOD isozymes were quite different from those in the culture medium. Cu/ZnSODI was the most abundant of the intracellular SOD isozymes (Fig. 4(a)); it was highly induced between 5 DAS and 15 DAS and then decreased. The other intracellular SOD isozymes showed different patterns. MnSODI, a unique intracellular isozyme, became detectable at 20 DAS, while the MnSODIII band disappeared by 20 DAS. Strong bands representing MnSODII and IV were present 25 DAS. MnSODII and IV isozymes were the major isozymes in the culture medium, and their levels gradually increased with cell growth. The FeSOD, Cu/ZnSODII, and MnSODV isozymes were present only in culture medium and were detectable from 20 DAS. These results indicate that strong induction of the MnSOD isozyme in cells and culture medium according to cell growth is related with oxidative stress, and different SOD isozymes are responsible to catalyze the scavenging of ROIs during cell cultures of sweetpotato. Vattanaviboon et al. (2003) demonstrated the induction of intracellular MnSOD in bacterial cells entering stationary phase. Moreover, intracellular MnSOD was reported to respond positively to salt stress (Gomez et al., 1999), manganese toxicity (Gonzalez et al., 1998), chilling stress (Lee et al., 1999), and drought (Wu et al., 1999).

As shown in Fig. 4(b), POD presents almost the same isozyme patterns, whereas isozyme levels were quite different in cells and culture medium during cell

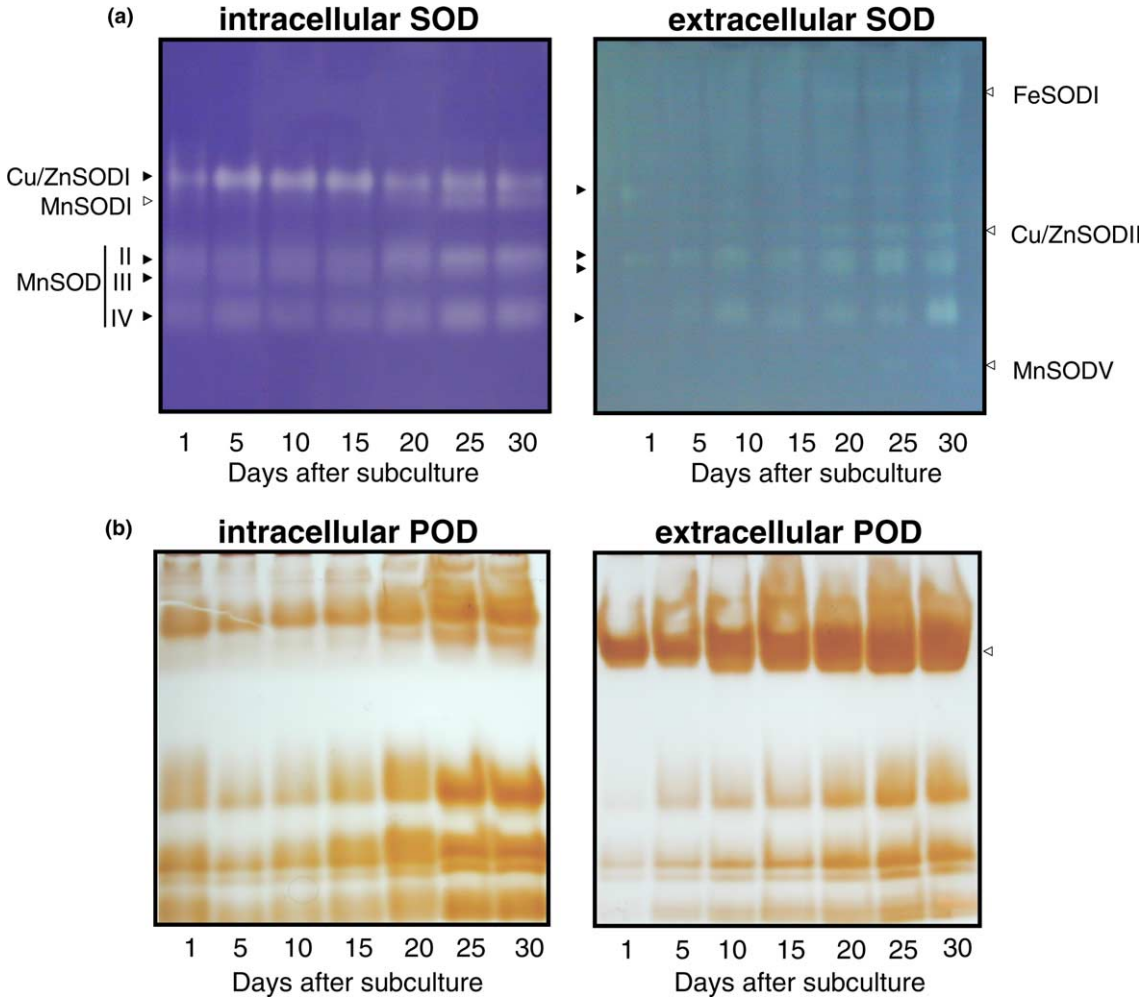

Fig. 4. Isozyme patterns of SOD and POD in suspension cultures of sweetpotato. (a) Native PAGE of SOD isozymes during cell growth. SOD activity was detected with a negative staining solution using NBT after electrophoresis; 10 μg of intracellular protein or 2 μg of extracellular protein concentrated from culture medium was loaded in each lane. ▶, Isozyme common to both cells and culture medium. ▷, Isozyme unique to cells or culture medium. (b) Native PAGE of POD isozymes during cell growth. POD activity was detected by benzidine staining after electrophoresis; 20 μg of intracellular protein or 1 μg of extracellular protein concentrated from cultured medium was loaded in each lane. ▷ major POD isozyme in culture medium.

growth. All intracellular POD isozymes increased evenly with cell growth. We previously isolated and characterized a major intracellular POD isozyme and indicated that the intracellular POD isozyme responds to environmental stresses (Huh et al., 1997). On the other hand, the extracellular POD isozyme that gave a strong band at the top of the native gel was the major POD isozyme in the culture medium; it will be interesting to further characterize this POD isozyme of sweetpotato. The alterations in SOD and POD isozyme levels observed here suggest that different intracellular and extracellular isozymes might modulate ROI levels during cell growth.

3. Conclusions

As a mechanism for coping with the deleterious effects of culture stress, rapid molecular responses have developed in cells to protect or repair damage by ROI and to protect against further exposure to the same and other forms of stress. The results presented here show that SOD, POD, and GPX are not only synthesized in cultured sweetpotato cells but are also secreted at high levels into the culture medium. Different intracellular and extracellular isozymes of sweetpotato might modulate ROI levels during cell growth. Further characterization of the extracellular antioxidant enzymes discovered in this work and isolation of their genes and promoters offer the opportunity to elucidate the antioxidant defense mechanism in response to oxidative stress.

4. Experimental

4.1. Plant material and cell cultures

The SP-47 cell line of sweetpotato suspension cultures (*I. batatas* (L) Lam. cv. White Star) was used (Kim et al., 1994). Cells (1 g fresh weight) subcultured at 14-day intervals were inoculated into 50 ml of MS basal medium (Murashige and Skoog, 1962) supplemented with 1 mg/l of 2,4-dichlorophenoxyacetic acid and 30 g/l of sucrose and cultured at 25 °C in the dark (100 rpm). Cells and medium were collected at 1, 5, 10, 15, 20, 25, and 30 days.

4.2. Preparation of extracts for enzyme assays and protein determinations

Suspension-cultured cells from three batches (150 ml) were centrifuged at 10,000*g* for 10 min at 4 °C. For extracellular antioxidant enzyme (SOD, POD, and GPX) determinations, the supernatant was concentrated 10–20-fold using an ultrafiltration filter (Millipore), dialyzed against 50 mM phosphate buffer (pH 7.0), and stored at −70 °C. The cells collected by centrifugation were weighed to determine the fresh weight and resuspended in 50 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA and 1% (w/v) PVPP for SOD analysis, in 0.1 M potassium phosphate buffer (pH 6.0) for POD analysis, or in 50 mM Tris–HCl buffer (pH 7.6) with 0.1 mM EDTA for GPX analysis. The cells were ground with a mortar and pestle. The suspensions were centrifuged again for 20 min at 4 °C, and supernatants were used as a source of intracellular enzymes (SOD, POD, and GPX). Protein contents were determined according to the method of Bradford (1976) using the Bio-Rad protein assay reagent.

4.3. Enzyme activities

The SOD activity was measured according to the method of McCord and Fridovich (1969) using xanthine, xanthine oxidase, and cytochrome c. One unit of SOD was defined as the amount of enzyme that inhibited the rate of ferricytochrome c reduction by 50%. The POD activity was assayed according to the method described by Kwak et al. (1995) using pyrogallol as a substrate. One unit of POD activity was defined as that forming 1 mg of purpurogallin from pyrogallol during 20 s at pH 6.0. The GPX activity was determined by following the decrease in absorbance of NADPH at 340 nm using glutathione reductase, glutathione (reduced, GSH), and cumene hydroxyperoxide at pH 7.6 according to Tappel (1978).

4.4. Native gel assay

Native polyacrylamide gel electrophoresis (PAGE) of SOD was performed on a 10% gel at 80 V and 4 °C for 5 h (Beauchamp and Fridovich, 1971). After electrophoresis, the gels were stained for SOD activity. The gel was incubated in the dark for 30 min in staining buffer (50 mM potassium phosphate buffer, pH 7.8, 0.026 mM riboflavin, 0.25 mM NBT, 0.2% TEMED) and then exposed to a light box until the SOD activity bands became visible. The SOD isozymes were differentiated by incubating the gel for 20 min in 50 mM potassium phosphate buffer, pH 7.8, containing either 3 mM KCN or 5 mM H_2O_2 before staining for activity. Cu/ZnSODs were inhibited by KCN and H_2O_2; FeSODs were resistant to CN^- but were inactivated by H_2O_2; MnSODs were resistant to both inhibitors. Native PAGE of POD was conducted on a 10% gel at 100 V and 4 °C for 2.5 h (Kim et al., 1994). After native PAGE, POD was stained with 1% benzidine and 1.5% H_2O_2.

Acknowledgements

This work was supported by Korean Research Foundation Grant (KRF-2003-041-F20019). We thank to Dr. Michung Suh for useful discussion and comments.

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