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Accumulation of salicylic acid-induced phenolic compounds and raised activities of secondary metabolic and antioxidative enzymes in *Salvia miltiorrhiza* cell culture

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ABSTRACT

The present work investigated the effects of salicylic acid (SA) on the accumulation of phenolic compounds and the activities of PAL, TAT, SOD, CAT and POD enzymes in the *Salvia miltiorrhiza* cell culture. When SA is applied to the cell culture, phenolic compounds will increase and PAL, TAT, SOD, CAT, and POD enzymes will become more active. The accumulations of phenolic compounds and the PAL activity were stimulated 8 h after the treatment with SA. The TAT activity was stimulated after 48 h. The resulting antioxidative enzymes' activities were greatly improved. SA elicitation on the phenolic acid accumulation was depended upon the application dosage and the time-duration. The suitable SA concentration for eliciting phenolic compound accumulations was 6.25–22.5 mg/L. The elicitation effect of SA on phenolic compound accumulations correlated with the PAL activity, but not with the TAT activity. This indicates that PAL may be the key enzyme for the biosynthesis of salvianolic acid B and caffeic acid. The raised PAL activity leads to the improvement of the quantity of phenolic compounds. This could be of particular significance by using plant cell culture systems for biotechnological production of plant secondary metabolites such as salvianolic acid B and caffeic acid.

1. Introduction

Plant secondary metabolites are unique sources for pharmaceuticals, food additives, flavors, and other industrial materials either as part of a final product or as a raw material (Zhao et al., 2005). To enhance the production of secondary metabolites, strategies such as treating with elicitors and invoking biotic and abiotic stresses have been used in both plant cell culture and intact plants (Zhang et al., 2004; Zhao et al., 2005). Elicitors refer to chemicals that can trigger physiological and morphological responses and secondary metabolism in plants (Benhamou, 1996). Elicitation causes a variety of defensive reactions and the accumulations of secondary metabolites (Yukimune et al., 1996; Zhang et al., 2004; Zhao et al., 2005).

Salicylic acid (SA) plays a key role in a plant's growth, development, and defense responses, and it is involved in some signal transduction systems to induce particular enzymes (Chen et al., 2006). As an elicitor, SA regulates the PAL enzyme activity, which as a biosynthetic enzyme catalyzes biosynthetic reactions for forming defensive compounds (Sgarbi et al., 2003; Solecka and Kacperska, 2003; Zhao et al., 2005) and SA regulates the protective enzymes such as SOD and POD, which together increase a plant's tolerance to environmental stresses (Mutlu et al., 2009; Shi and Zhu, 2008; Shim et al., 2003; Thulke and Conrath, 1998).

Oxidative burst is a common early response of plants to elicitor treatments, which induces the enhancement of plant antioxidative enzyme activity to scavenge the generated active oxide species (AOS) (Chen et al., 2006; Shim et al., 2003; Thulke and Conrath, 1998). SOD is a major scavenger of superoxide radicals and the enzymatic action results in the formation of H_2O_2 that is then converted to H_2O and O_2 by POD and CAT (Alscher et al., 2002; Takahashi and Asada, 1983). SA can increase SOD and POD activities with the resulting increase of AOS scavenging to protect plants from being injured (Mutlu et al., 2009; Shi and Zhu, 2008). There is an evidence that SA will decrease the CAT activity (Dat et al., 2000; Shi et al., 2006; Shi and Zhu, 2008; Shim et al., 2003), but in Kentucky bluegrass (He et al., 2005) and in wheat (Agarwal et al., 2005), SA actually increased the CAT activity. The relationships and the interactions between SA, the protective compounds biosynthesis, and the related plant enzymes are not fully understood.

The *Salvia miltiorrhiza* Bunge's roots and rhizomes have been used as a traditional Chinese herbal drug (known as Danshen) for removing blood stasis, alleviating pain, promoting the circulation of blood, promoting menstruation, tranquilizing the brain, and treating cardiovascular and cerebrovascular diseases (China Pharmacopoeia, 2005). Danshen contains two groups of bioac-

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tive constituents that are lipid-soluble diterpenes (tanshinones) and water-soluble phenolic compounds (Chen and Chen, 1999). Researchers are exploring new benefits from the tanshinones accumulation (Chen and Chen, 1999; Ge and Wu, 2005a, 2005b; Shi et al., 2007; Wu et al., 2007; Yan et al., 2005), but so far only preliminary work has been done on phenolic compound accumulations (Yan et al., 2006). The early works on phenolic compounds have shown that there are significant bioactivities such as antioxidant, anti-ischemia reperfusion, and antithrombotic effects (Chen et al., 2000; Li, 1997; Yan et al., 2006). Among the phenolic compounds, salvianolic acid B is an index chemical in the quality control of Danshen (China Pharmacopoeia, 2005) and it shows a great intensity for free radical scavenging and antioxidance (Huang and Zhang, 1992). Rosmarinic acid can be considered as the condensation of caffeic acid and 3,4-dihydroxyphenyllactic acid and it is the crucial precursor for salvianolic acid B biosynthesis (Zhao et al., 2004). The caffeic acid part is formed from phenylalanine, while the 3,4-dihydroxyphenyllactic acid part is formed from tyrosine (Ellis and Towers, 1970; Petersen and Simmonds, 2003; Zhao et al., 2004). PAL is the key enzyme in the phenylalanine-derived pathway, and tyrosine aminotransferase (TAT) is the first enzyme in the tyrosine-derived pathway (Dixon and Paiva, 1995; Petersen et al., 1993). There is evidence that the PAL activity was suppressed, while the TAT activity was enhanced by the elicitation of Ag^+ and yeast extract in the *S. miltiorrhiza* hairy root culture (Yan et al., 2006).

The primary aim of this work was to investigate the effects of SA elicitation in the *S. miltiorrhiza* cell cultures on the accumulation of phenolic compounds (salvianolic acid B and caffeic acid). The secondary aim was to assess the effects of SA on the secondary metabolism enzymes (PAL and TAT) and on the antioxidative enzymes (SOD, CAT and POD).

2. Materials and methods

2.1. Cell culture and elicitor treatment

A germfree seedling leaf of *S. miltiorrhiza* was cut into 0.5 cm × 0.5 cm segments and inoculated on the autoclaved MS media supplemented with 100 mg/L of myoinositol, 30 g/L of sucrose, and 4.2 g/L of agar with a pH 5.8. The segments were grown at 24 ± 2 °C under a 12 h photoperiod for induction of calli.

The investigation of the elicitation effect of SA was conducted in liquid shake-flask cultures (100-mL Erlenmeyer flasks) on an orbital shaker at 125 rpm. The calli were transferred to these flasks and maintained on liquid hormone-free MS media supplemented with 30 g/L of sucrose and different concentrations of SA at 25 °C in darkness. Each flask was filled with 30 mL of MS medium and inoculated with 1.3 g of fresh calli from 20-day-old flask cultures. The calli were transferred to newly prepared media every 20 days for sub-culturing during the SA treatment. At the end of the period, the calli were harvested from the cultured media through the steps of filtering, blotting dry with paper towels to yield fresh weight (FW), and drying at 40 °C in a vacuum oven until constant dry weight (DW) was obtained.

2.2. Analysis of phenolic compounds

The phenolic compounds were extracted in an ultrasonic bath as previously described by Dong et al. (2010). The extracted solution was filtered and concentrated *in vacuo*. The concentrated solution was diluted with distilled water and then acidified to pH 2. The obtained liquid was extracted with ethyl acetate. Then the extract was evaporated. Afterwards 10 mL of methanol was added and the solution was filtered through a millipore filter (0.45 μm). The resulting filtrate was analyzed by high performance liquid chromatography [Shimadzu, model SCL-10A*VP*™ equipped with UV/Vis absorbance detector (SPD-10AVP), 150 mm × 4.6 mm i.d. 5 μm shim-pack column (VP-ODS), and infusion pump (LC-ATVP)]. Salvianolic acid analysis was conducted under the following conditions: mobile phase, methanol–2% glacial acetic acid (40:60, v/v); flow rate, 1 mL/min; injection amount, 5 μL; detection wavelength, 281 nm, and column temperature, 30 °C. The analysis of caffeic acid was conducted under similar conditions: mobile phase, methanol–2% glacial acetic acid (24:76, v/v); flow rate, 1 mL/min; injection amount, 5 μL; detection wavelength, 319 nm, and column temperature, 30 °C.

2.3. Analysis of PAL and TAT activities

PAL activity was measured as described by Solecka and Kacperska (2003) with some modifications. Using a mortar and pestle, 2 g of calli were homogenized with 5 mL of extraction buffer. The homogenate was filtrated through four layers of cheesecloth and centrifuged at 12,000 × *g* for 15 min at 4 °C. The supernatant was used as a crude enzyme. The reaction mixture (3 mL) contained 0.5 mL of the crude enzyme, 16 mM of L-phenylalanine, 50 mM of Tris–HCl buffer (pH 8.9), and 3.6 mM of NaCl. The mixture was incubated at 37 °C for 60 min and the reaction was stopped by 500 μL of 6 M HCl. The reaction mixture was then centrifuged for 10 min at 12,000 × *g*. The absorbance was measured at 290 nm before and after incubation.

TAT activity was determined by the method described by Yan et al. (2006). Using a mortar on ice with a chilled pestle, fresh calli were grounded with the extraction buffer (0.25 g FW/mL) and this mixture was centrifuged at 13,000 × *g* at 4 °C for 25 min to obtain a solid-free crude enzyme extract. A 50 μL enzyme extract was added to a reaction solution containing 6.0 mM of L-tyrosine, 10 mM of a-ketoglutarate, 0.05 mM of pyridoxal phosphate, and 42.5 mM of glycyglycine at pH 9.0. After mixing, the reaction mixture was incubated at 30 °C for 30 min. The reaction was stopped by 20 μL of 10 M KOH. Then the TAT activity was determined by measuring the initial (0 min) and final absorbance (30 min) of the reaction solution against a reagent blank at 331 nm.

2.4. Analysis of SOD, CAT and POD activities

SOD activity was determined by measuring its ability to inhibit the auto-oxidation of pyrogallol as described by Marklund and Marklund (1974). The SOD was extracted at 4 °C from 1 g FW finely ground calli by 10 mL of a cold solution of 1.33 mM of diethylenetriamine penta acetic acid in 50 mM of potassium phosphate buffer (pH 7.8). After the homogenate was centrifuged twice at 4 °C for 15 min at 27,000 × *g*, the supernatant was retained for the SOD assay. The reaction mixture contained 1 mL of 0.6 mM pyrogallol, 1.5 mL of 100 mM Tris–HCl buffer (pH 8.2), 0.5 mL of 6 mM EDTA, and 0.1 mL of enzyme extract. The rate of pyrogallol auto-oxidation was measured from the increase in absorbance at 420 nm in a spectrophotometer after an interval of 15 s up to 2 min. One unit of SOD activity was defined as the amount of enzyme that would inhibit 50% of pyrogallol auto-oxidation.

POD activity was measured by monitoring the increase in absorbance at 470 nm in 50 mM of phosphate buffer (pH 5.5) containing 1 mM of guaiacol, 0.5 mM of H_2O_2, and 0.1 mL of enzyme extract. One unit of POD activity was defined as the amount of enzyme that caused an increase in absorbance of 0.01 of material per min (Upadhyaya et al., 1985).

CAT activity was measured by the method as described by Chakraborty and Tongden (2005) with modifications. CAT was extracted from 1 g of finely ground calli in 10 mL of cold 100 mM potassium phosphate buffer (pH 6.8) at 4 °C. The homogenate

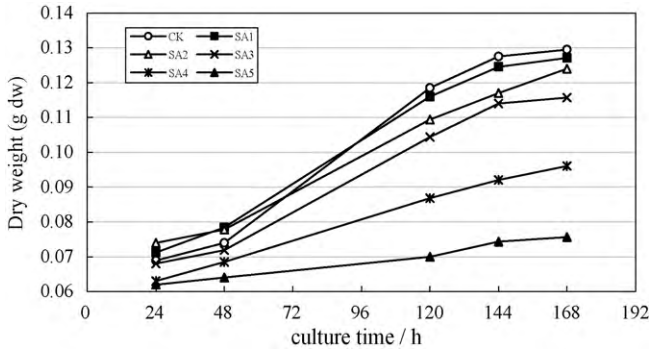

Fig. 1. Effects of SA application on the *S. miltiorrhiza* cell growth. The lines for SA1–SA5 represent the SA concentrations of 3.125, 6.25, 12.50, 22.5, and 25.0 mg/L, respectively. The value of dry weight is the mean of triplicates.

was centrifuged and the supernatant was used to determine the CAT activity. The CAT reaction mixture (3 mL) contained 50 mM of Na–phosphate buffer pH 7.0, 15 mM of H_2O_2, and 0.1 mL of enzyme extract. Changes in the absorbance of the reaction solution at 240 nm were recorded after every 20 s. Enzyme activity was expressed as ΔA 240 min^{-1} g^{-1} FW.

2.5. Statistics

Statistical analysis was carried out by using analysis of variance (ANOVA) and by using SAS software (SAS Institute, Cary NC). Differences were separated out by using the least significant difference (LSD) test at a 0.05 probability level.

3. Results and discussion

3.1. Effects of salicylic acid on cell growth

The growth rates of cell cultures were investigated at 24, 48, 120, 144, and 168 h after the treatments with different concentrations of SA. From 0 to 48 h, the cell cultures grew slowly and there were no significant differences in the weight of each treated group and the control. From 48 to 120 h, the weights of cell cultures increased rapidly, but at the 120 h point, the growth rate began to taper off (Fig. 1). On the other hand, the growth rates were significantly inhibited by the high concentrations of SA (22.5–25.0 mg/L), but the low concentrations (3.125–12.5 mg/L) did not show any significant impact. 168 h after the SA treatment, the weight of SA5 (25.0 mg/L) was only 58.38% of the control, however, the weight of SA3 (12.5 mg/L) was 89.34% of the control.

3.2. Effects of salicylic acid on the accumulation of phenolic acids in the cell cultures

The time lines of the SA elicitor on the accumulations of the phenolic acids (salvianolic acid B and caffeic acid) in the treated cell cultures are shown in Fig. 2. The phenolic compounds were measured after an application of 22.5 mg/L of SA to evaluate the effects of SA on the induction of salvianolic acid B and caffeic acid. An application of 22.5 mg/L of SA had an obvious effect on the accumulations of salvianolic acid B (Fig. 2A) and of caffeic acid (Fig. 2B). After adding SA to each cultured medium, the concentrations of both phenolic acids were dramatically increased compared to the control. After the SA treatments, two peaks of each phenolic acid accumulations appeared. The first salvianolic acid B peak appeared at the 8 h point and the caffeic acid appeared at the 4 h point. The second salvianolic acid B peak appeared at the 96 h point and the second caffeic acid peak appeared at the 80 h point. In addition, the

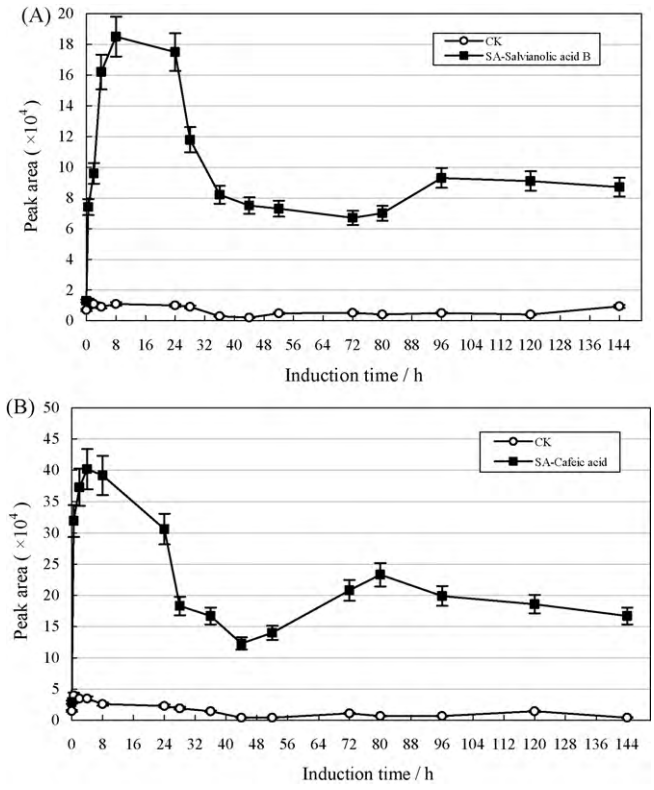

Fig. 2. Effects of SA elicitor on the extracellular concentration of salvianolic acid B (A) and caffeic acid (B) in the *S. miltiorrhiza* cell cultures. The SA concentration is 22.5 mg/L. Each value of the peak area is the mean of triplicates (±S.D.).

caffeic acid responded faster to the presence of SA than salvianolic acid B, which indicated that caffeic acid provided a stronger protective effect to the stressed cell culture than salvianolic acid B. This may be due to the fact that caffeic acid is an upstream product in the biosynthesis pathway of salvianolic acid B (Zhao et al., 2004).

The effects of SA with different concentrations on the accumulations of phenolic acids in the *S. miltiorrhiza* cell cultures are shown in Fig. 3. SA obviously affected the accumulations of salvianolic acid B (Fig. 3A) and of caffeic acid (Fig. 3B) in these cell cultures. Both phenolic acids accumulations were significantly increased 8 and 96 h after the applications of 3.125–25.0 mg/L of SA, but were significantly less with 32.0–50.0 mg/L of SA. 96 h after the treatments with 3.125–25.0 mg/L of SA, the concentration of the phenolic acids decreased significantly compared to the amount 8 h after the treatments, but were still higher than that of the control.

To summarize, a low concentration (such as 3.125–22.5 mg/L of SA) treatment increased the accumulations of the phenolic acids in the *S. miltiorrhiza* cell cultures, whereas a high concentration (such as 32.0–50 mg/L of SA) treatment resulted in a decreased accumulations of the phenolic acids. Thus the elicitation effect was dependent on both the SA dosage and the elapsed time. The best combination was 6.25–12.5 mg/L of SA and 8 h.

The present work has shown that SA elicitation resulted in a decrease in the biomass (Fig. 1) while increasing the phenolic compound accumulations. Chen and Chen (1999) had observed the same behavior in their work with Ti transformed *S. miltiorrhiza*. This may indicate that elicitation with SA switched the cells partially from primary to secondary metabolism.

3.3. Effects of salicylic acid on the activities of PAL and TAT

It has been reported that rosmarinic acid is a crucial precursor for forming salvianolic acid B in *S. miltiorrhiza* (Zhao et al.,

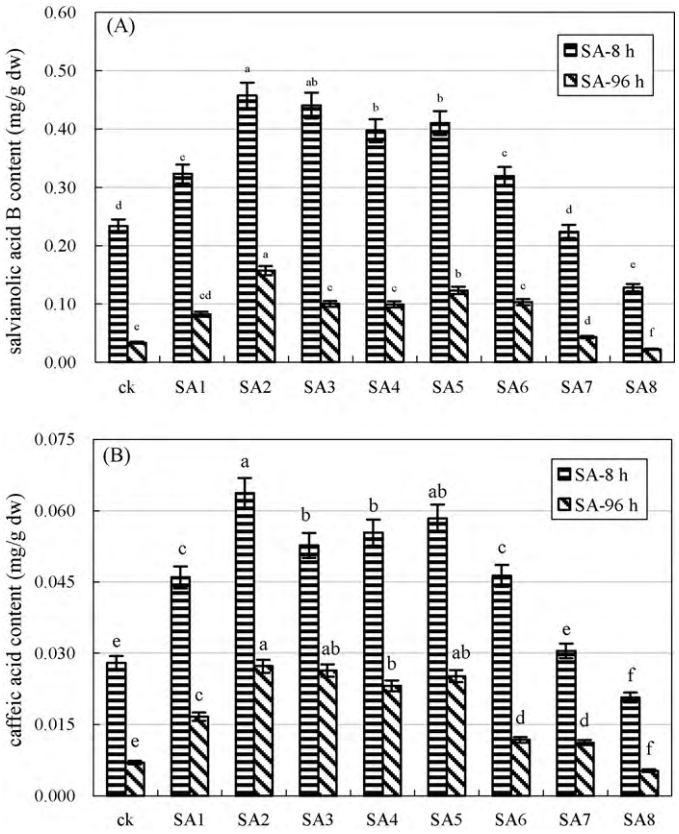

Fig. 3. Effects of SA elicitor on the accumulation of salvianolic acid B (A) and caffeic acid (B) in the *S. miltiorrhiza* cell cultures. The columns for SA1–SA8 represent the SA concentrations of 3.125, 6.25, 12.50, 18.75, 22.5, 25.00, 32.00, and 50.00 mg/L, respectively. Each value of the content of salvianolic acid is the mean of the total phenolic acid in the intracellular and medium of triplicates (±S.D.). Different lowercase letters represent the significant differences between the concentrations of salvianolic acid B (A) and caffeic acid (B) 8 and 96 h after SA elicitation at the level of 5%.

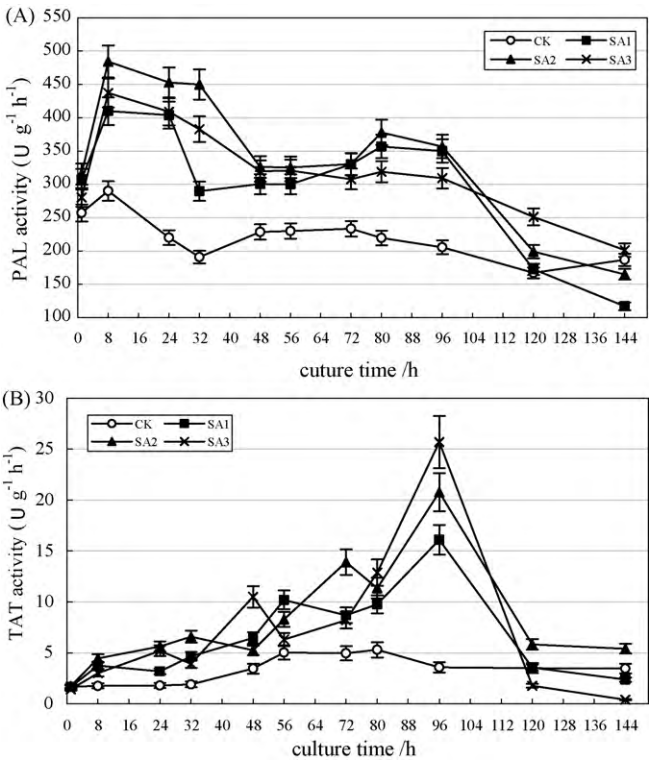

Fig. 4. Effects of SA elicitor on the activity of PAL (A) and TAT (B) of the *S. miltiorrhiza* cell cultures. The lines for SA1–SA3 represent the SA concentrations of 3.125, 6.25, and 12.50 mg/L, respectively. Each value is the mean of triplicates (±S.D.).

2004). The biosynthesis of rosmarinic acid has been demonstrated the involvement of both the phenylpropanoids pathway and the tyrosine-derived pathway (Ellis and Towers, 1970; Petersen and Simmonds, 2003). PAL is a branch point enzyme between the primary and the secondary metabolism, and is a key enzyme for regulating the influx of phenylalanine to the biosynthesis of phenolic compounds (Dixon and Paiva, 1995). Evidence has shown that the PAL activity could be induced by SA elicitation in parsley, citrus, and grapes (Chen et al., 2006; Lafuente et al., 2004; Thulke and Conrath, 1998), which would result in the accumulation of plant secondary metabolites (Janas et al., 2002; Sgarbi et al., 2003; Solecka and Kacperska, 2003; Zhao et al., 2005). The present work also showed that the PAL activity could be induced by SA elicitation in the *S. miltiorrhiza* suspension cell culture. Furthermore, the effect of SA elicitation dosage and the time-duration on the PAL activity were nearly the same as for the phenolic acids (Fig. 2). The PAL activity increased to the first peak at the 8 h point, and after this came a gradual decrease and the second peak appeared 80 h after the SA treatment (Fig. 4A). The best dosage of SA elicitation was with a treatment of 6.25 mg/L, which resulted in the greatest phenolic acids accumulations (Fig. 3, SA2). This work showed that both the PAL activity and phenolic acid accumulations were enhanced dramatically after the SA treatment, indicating that PAL is the key enzyme in regulating the SA elicited phenolic acid accumulations in the *S. miltiorrhiza* cell cultures. Yet very little is known or understood about the role that SA plays in regulating PAL activity and phenolic compound biosynthesis (Chen et al., 2006) and about the relationships among SA, PAL, and phenolic compounds in biosynthesis.

TAT is the first enzyme in the tyrosine-derived pathway for the biosynthesis of rosmarinic acid (Ellis and Towers, 1970; Yan et al., 2006). In our work, the TAT activity was enhanced by the elicitation of SA, which is corresponding with the results as the elicitation of Ag^+ and yeast extract in the *S. miltiorrhiza* hairy root culture (Yan et al., 2006). The TAT activity rose slightly above that of the control cell culture and gradually increased to a peak at the 96 h point after which a rapid decline occurred (Fig. 4B). The maximal effect was achieved with the 12.5 mg/L treatment at the 96 h point (Fig. 4B, SA3). The effect of SA on the TAT activity is also related to the dosage and the time-duration. After 80 h elicitation, the TAT activity was enhanced significantly (Fig. 4B), but the phenolic acid accumulations were not enhanced significantly (Fig. 2). The TAT activity does not correlate to the same level of the PAL activity and the phenolic acid accumulations with the elicitation of SA in the *S. miltiorrhiza* suspension cell culture.

3.4. Effects of salicylic acid on the activities of SOD, POD and CAT

An important early response of plant cells to elicitor treatment is the oxidative burst associated with the increase amounts of AOS (Thulke and Conrath, 1998; Chen et al., 2006; Zhao et al., 2005). When a plant is exposed to stressful conditions, the plant's antioxidant system plays a crucial protective role by increasing antioxidative enzymatic activity or by enhancing certain compounds (Fecht-Christoffers et al., 2003; Shi et al., 2006). Of the antioxidative enzymes, SOD constitutes the first line of defense against AOS and is a major scavenger of the superoxide (Alscher et al., 2002; Takahashi and Asada, 1983). POD is known to decompose H_2O_2 by the oxidation of the phenolic compounds and prevent the lipid peroxidation of the membranes (Chakraborty and Tongden, 2005). Evidences showed that SA increased the SOD and POD activi-

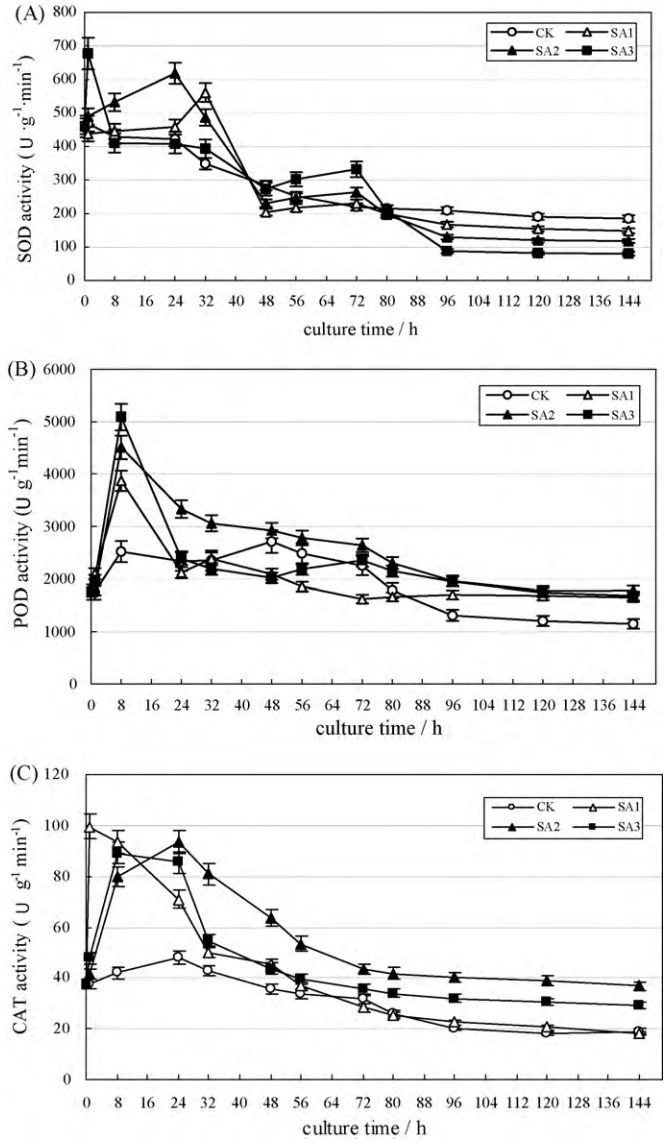

Fig. 5. Effects of SA elicitor on the activity of SOD (A), POD (B) and CAT (C) of the *S. miltiorrhiza* cell cultures. The lines for SA1–SA3 represent the SA concentrations of 3.125, 6.25, and 12.50 mg/L, respectively. SA elicitor was fed to the medium of culture on day 20 post-inoculations. Each value is the mean of triplicates (±S.D.).

ties (Mutlu et al., 2009; Shi and Zhu, 2008). In our work, SOD activity was significantly increased during 0–32 h and peaked at different times with the larger concentrations of SA elicitation invoking the peaks to appear earlier (Fig. 5A). The highest SOD activity occurred at the 1 h point with 12.50 mg/L (Fig. 5, SA3). The peak for 6.25 mg/L occurred at the 24 h point (Fig. 5, SA2) and the peak for 3.125 mg/L occurred at the 32 h point (Fig. 5, SA1). This suggests that the higher the concentration of the SA application, the earlier and the higher the SOD activity peak would appear. These results indicated that SOD responded rapidly in order to protect a plant from the damaging exogenous SA. POD activity peaked at the 8 h point (Fig. 5B). From 8 to 24 h, the decrease was rapid. After the 24 h point, the decrease was slower and the activity level clustered around the control with some having even lower activity levels. Both the SOD and POD activities were enhanced rapidly after applications of SA, which indicates that the cultured cells responded by stimulating the antioxidant system to protect the plant from any injuries.

Evidences showed that H_2O_2 mediated the isoflavonoid glyceollin accumulations in soybeans (Guo et al., 1998), and $O_2^{\bullet-}$ mediated the phytoalexin accumulations in parsley cell cultures (Jabs et al., 1997). In the present work, SA simultaneously increased the accumulations of phenolic compounds and increased the activities of the H_2O_2 scavenging enzymes. This was consistent with the phenolic acid accumulations in the cell culture elicited by SA. These results suggested that some relationships might exist between H_2O_2 and the secondary metabolism in the *S. miltiorrhiza* cell culture. It is still not clear how the SA mechanisms controlled the antioxidative enzyme activities and mediate the secondary metabolite production in the presence of AOS.

While SOD and POD activities are enhanced, the CAT activity may decrease as is the case for tobacco (Dat et al., 2000), *Cucumis sativa* (Shi et al., 2006), rice (Shim et al., 2003), and cucumber (Shi and Zhu, 2008) or may increase as is the case for Kentucky bluegrass (He et al., 2005) and wheat (Agarwal et al., 2005). Mutlu et al. (2009) worked with wheat cultivars where some samples were enhanced by 0.1 mM of SA while other samples were slightly reduced by 0.01 mM of SA. For the *S. miltiorrhiza* cell cultures, this work showed that CAT activity increased dramatically after the SA treatment and peaked at different times based on the SA concentrations. CAT activity peaked during 0–32 h with the peaks occurring based on the SA dosage amount. During 32–72 h period, CAT activity decreased almost to the level that matched the control. Although CAT had the same behavior as the SOD for having peaks in reaction to the SA dosage amount, the difference is that when SOD had a second peak at the 72 h point, CAT did not. Instead the 72 h point marked the end of the steep decline and from this point onward the activity levels did not vary significantly (Fig. 5C). These results suggest that the effect of SA on CAT activity may depend upon the plant species, the applied dosage and the amount of elapsed time of SA.

4. Conclusion

The present work showed that SA enhanced the secondary metabolism of the PAL and TAT enzymes and the antioxidative activities of the SOD, CAT, and POD enzymes. In addition, this work showed that SA could elicit the accumulations of phenolic compounds. However, the SA elicitation effect is depended upon the SA concentration and the time-duration. The SA-induced phenolic compound accumulations are related to the PAL activity and not to the TAT activity. This suggests that in the *S. miltiorrhiza* cell cultures PAL may be the key enzyme involved in the biosynthesis of both salvianolic acid B and caffeic acid. The SA application stimulated the PAL activity, which lead to the enhanced accumulations of phenolic compounds. This could be of particular significance in using plant cell culture systems for the biotechnological production of both salvianolic acid B and caffeic acid from the *S. miltiorrhiza* cell cultures. As promising as these preliminary results may be, the relationships between SA, secondary metabolism, and the related enzymes are far from being completely understood and further investigations are needed to clarify the underlying mechanisms.

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