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Biosynthesis and Metabolism of Vitamin C in Suspension Cultures of *Scutellaria baicalensis*

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The concentrations of L-ascorbic acid (AsA, ascorbate, vitamin C) and its biosynthetic and metabolically-related enzymes such as L-galactono-1,4-lactone dehydrogenase (GLDase), ascorbate peroxidase (APX), and ascorbate oxidase (ASO) were investigated in suspension cultures of *Scutellaria baicalensis*. Cells growing from 4 days after subculture (DAS) to 9 DAS and from 16 DAS to 19 DAS showed a diauxic growth, and then growth rapidly decreased with further culturing. The AsA content slowly increased to 19 DAS, reached a maximum at 21 DAS (ca 120 μg/g dry cell wt), and then rapidly decreased with further culturing. GLDase and ASO activity were well correlated with the cell growth curve, showing a maximum at 19 DAS, whereas APX activity showed a good correlation with the changes in AsA content, showing a maximum at 21 DAS. The total ascorbate contents (reduced form, AsA, and oxidized form, dehydroascorbate, DHA) were markedly enhanced at 10 DAS when L-galactose and L-galactono-1,4-lactone (25 mM) were added to SH medium supplemented with 20 g/l sucrose at 9 DAS, by 5.5 and 6.8 times, respectively. DHA composed more than 90% of the total ascorbate contents in suspension cultures of *S. baicalensis*, even though the ratio of reduced to oxidized form slightly varied with cell growth stage. The results indicate that L-galactose and L-galactono-1,4-lactone are effective precursors of AsA in cell cultures of *S. baicalensis*, and that *in vitro* cultured cells provide suitable biomaterials for the study of biosynthesis and metabolism of AsA.

Keyword: Ascorbic acid (vitamin C), Biosynthesis, L-Galactose, *Scutellaria baicalensis*, Suspension culture.

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Introduction

Oxidative stress is a major cause of damage in plants exposed to stressful environmental conditions and results from the cellular damage caused by reactive oxygen species (ROS) generated in cells. Plants have developed antioxidative defense systems to cope with these ROS, including antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and low molecular mass antioxidants such as L-ascorbic acid (AsA, ascorbate, vitamin C), glutathione (GSH), and α-tocopherol (vitamin E) (Alscher and Hess, 1995; Lee and Kim, 1998; Lee *et al.*, 1999)

AsA is found in millimolar concentrations in plants, but little is known about its biosynthesis and role in adaptation of plants against environmental stress (Smirnoff, 1996; Smirnoff and Pallanca, 1996; Noctor and Foyer, 1998; Wheeler *et al.*, 1998). Recently, Conklin *et al.* (1996) reported that the ozone-sensitive mutant of *Arabidopsis thaliana* contained only 30% of the normal AsA concentration, and the mutant showed hypersensitivity to both sulfur dioxide and ultraviolet-B radiation, indicating that AsA might play an important role in defense against various biotic and abiotic environmental stresses.

If the metabolism desired is expressed in cultured cells, suspension cultures of undifferentiated plant cells provide ideal materials for *in vitro* studies of plant metabolism because of their ease in handling and apparent uniformity. In particular, cultured plant cells are considered to be grown under high oxidative stress conditions, which suggests that plant cell cultures are an efficient system for the study of antioxidative mechanisms and the production of useful antioxidants (Takeda *et al.*, 1990; Kwak *et al.*, 1995; 1996). In previous studies, some antioxidant enzymes such as POD, SOD, and CAT were characterized in various plant cell lines (Kim *et al.*, 1994; You *et al.*, 1996; Jang *et al.*, 1997). The possible mass production of POD and the physiological role of each isoenzyme have been

investigated in suspension cultures of sweet potato in terms of cellular adaptation against environmental stresses. Recently, we described the levels of AsA in various plant cell lines and the changes in AsA content during growth of callus cultures of *Scutellaria baicalensis* (Ahn *et al.*, 1998). In this paper, we describe the changes in AsA contents and its biosynthetic and metabolically-related enzymes in suspension cultures of *S. baicalensis*. In addition, the effects of possible precursors on the biosynthesis of AsA and dehydroascorbate (DHA) are discussed.

Materials and Methods

Plant cell materials The *Scutellaria baicalensis* cell line (KCTC plant cell line 10159) maintained at the Korean Collection for Type Cultures (KCTC), Genetic Resources Center, Korea Research Institute of Bioscience and Biotechnology, was used in this study for its high yield of AsA (Ahn *et al.*, 1998). One gram (fr. wt) of cells subcultured at 15 d intervals was inoculated into 50 ml of SH (Schenk and Hildebrandt, 1972) basal medium supplemented with 2 mg/l P-chlorophenoxyacetic acid, 0.5 mg/l 2,4-dichlorophenoxyacetic acid, 0.1 mg/l kinetin, and 30 g/l sucrose in a 300-ml Erlenmeyer flask at 25°C in the dark (100 rpm).

Analysis of AsA and DHA by HPLC Endogenous AsA was extracted and analyzed by the method of Graham and Annette (1992) with a slight modification. Cells were homogenized on ice with a mortar and a pestle in 62.5 mM metaphosphoric acid (1:2, w/v). The homogenate was centrifuged at 12,000 × *g* for 20 min at 4°C. For the analysis of total AsA, the oxidized AsA (DHA) in the sample was reduced by a slightly modified method of Law *et al.* (1983). A solution of 60 mM DTT was added to the extract and incubated for 24 h at 25°C. Samples with or without DTT treatment were filtered through a 0.5 μm FH-type Millipore filter. The filtrate was loaded onto an aminex HPX-87H ion exclusion column (300 × 7.8 mm, Bio-Rad) connected to a Spectra-Physics HPLC system, and eluted with 4.5 mM H_2SO_4 at a flow rate of 0.5 ml per min. The elution of AsA was monitored at 245 nm. Retention times of AsA and DHA were found to be 12.3 and 10.9 min, respectively. Quantitative analysis was carried out by comparing the peak areas of the samples with those of the authentic AsA. DHA was determined as the difference between total AsA contents after DHA reduction and AsA contents of the original sample.

Analysis of enzyme activity GLDase activity was measured by the method of Oba *et al.* (1995). Cells were homogenized on ice with a mortar in 0.1 M potassium phosphate buffer containing 0.4 M sucrose and 30 mM mercaptoethanol (pH 7.4, 1:2, w/v). The homogenate was centrifuged at 12,000 × *g* for 20 min at 4°C and the supernatant was used immediately for enzyme assays. The assay mixture contained 10 mM potassium phosphate buffer, cytochrome c (1.5 mg/ml), and 4.2 mM L-galactono-1,4-lactone (L-Gl). Enzyme activity was assayed by measuring the L-Gl-dependent reduction of cytochrome c at 550 nm. One unit of enzyme activity was defined as the amount of enzyme that could oxidize 1 μM of L-Gl per min. APX activity was measured by the method of Torsethaugen *et al.* (1997). Sodium phosphate buffer (90 mM) containing 8% glycerol, 1 mM EDTA, and 5 mM AsA was used for extraction. The assay mixture contained 50 mM potassium-phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.1 mM hydrogen peroxide, and 0.5 mM AsA. APX activity was calculated from the decrease in absorbance at 290 nm (an absorbance coefficient of 2.8 per mM per cm) as AsA oxidized for the first 30 s of the reaction. ASO activity was measured by the method of Oberbacher *et al.* (1963). Potassium phosphate buffer (0.1 M, pH 6.5) was used for enzyme extraction and enzyme activity was determined by measuring AsA oxidation at 265 nm in 0.1 M potassium phosphate buffer (pH 5.6) with 0.5 μM AsA. One unit of ASO activity was defined as that oxidizing 1 μM of AsA to DHA per min.

Precursor treatment Sucrose, D-glucose, D-fructose, D-mannose, L-galactose, and L-galactono-1,4-lactone were used as putative precursors of AsA biosynthesis. Each precursor (25 mM) was added to cultures in the SH medium containing 20 g/l sucrose as a basal carbon source at 3, 10, 17, and 24 d after subculture. After incubation for 24 h, AsA content was analyzed by HPLC.

Results

Changes in AsA content during cell cultures The changes in AsA content in suspension cultures of *Scutellaria baicalensis* are shown in Fig. 1A. Cells grew slowly from 4 days after subculture (DAS) to 9 DAS. After a rather slow growth stage from 9 DAS to 16 DAS, cells displayed a final growth spurt from 16 DAS to 19 DAS evidenced by the diauxic growth curve, and then rapidly decreased by massive cell lysis with further culturing. AsA content was relatively high just after subculture. After slightly decreasing to 2 DAS, AsA content increased slowly from 2 DAS to 19 DAS, reached a maximum at 21 DAS (ca 120 μg/g dry cell wt), and then rapidly decreased with further culturing.

Changes in GLDase, APX, and ASO activities during cell culture The relative changes of GLDase activity, an enzyme that catalyzes the final step in the biosynthesis of AsA, and two metabolic enzymes of AsA, APX and ASO, are shown in Fig. 1B. GLDase activity converting L-galactono-1,4-lactone (L-Gl) to AsA increased in proportion to cell growth up to 19 DAS (ca 1584 units/mg protein, the highest activity), equivalent to the maximum growth, and rapidly decreased with further culturing. ASO activity converting AsA to dehydroascorbate (DHA) showed fluctuations similar to that of GLDase during cell cultures, with highest activity at 19 DAS (ca 618 units/mg protein). Meanwhile, APX activity, converting AsA to monodehydroascorbate, showed a similar pattern to the AsA content, which was slightly different than those of GLDase and ASO. APX activity remained at a high level from subculture to 16 DAS (ca 356 units/mg protein), rapidly increased to 21 DAS (ca 696 units/mg protein, the highest activity), and then decreased beyond this point.

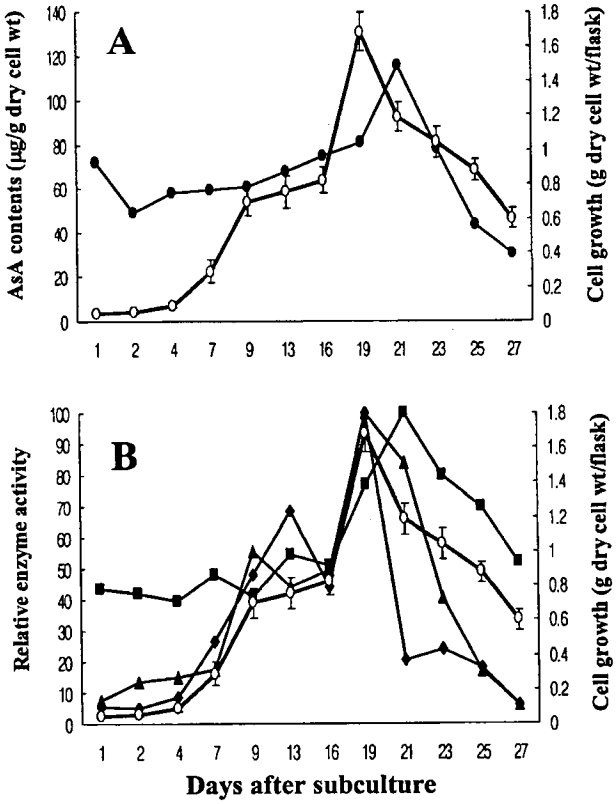

Fig. 1. Changes in vitamin C (AsA) content and its biosynthetic and metabolically-related enzymes during suspension cultures of *Scutellaria baicalensis* in SH basal medium supplemented with 30 g/l sucrose. **A.** Time course of cell growth and AsA content on the basis of g dry cell wt; **B.** Relative values of each enzyme activity against the highest activity. Data of cell growth are means ± S.E. of three replications. Similar results of enzyme activity were obtained in two other independent experiments. -○-, cell growth (g dry wt/flask); -●-, AsA content (μg/g dry cell wt); -◆-, galactono-1,4-lactone dehydrogenase; -▲-, ascorbate oxidase; -■-, ascorbate peroxidase.

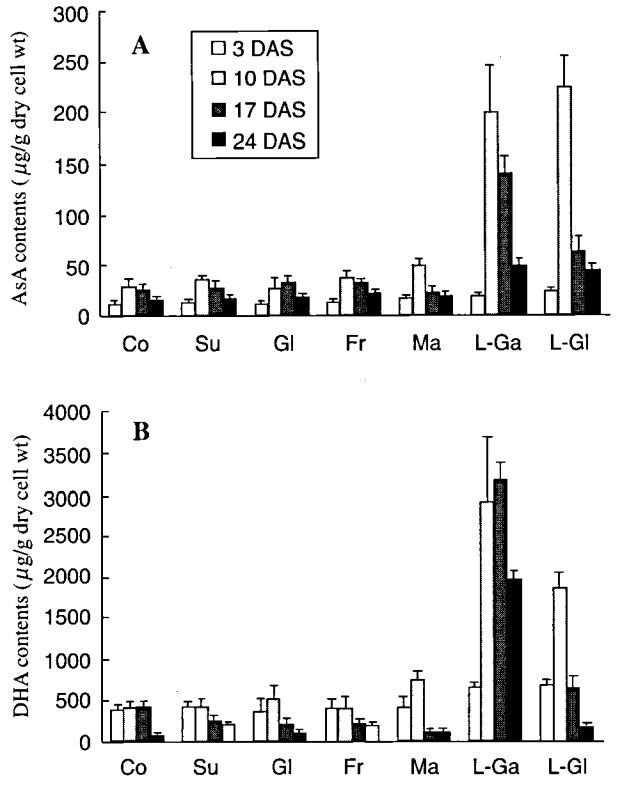

Fig. 2. Effects of various possible precursors for the biosynthesis of vitamin C (AsA) on the contents of AsA and dehydroascorbate (DHA) in suspension cultures of *Scutellaria baicalensis* in SH basal medium supplemented with 20 g/l sucrose. Each possible precursor (25 mM) was added to the medium at 3, 10, 17, and 24 days after subculture (DAS), and the contents of AsA and DHA were analyzed by HPLC one day one after treatment by HPLC. Co, Su, Gl, Fr, Ma, L-Ga, and L-Gl represent control, sucrose, glucose, fructose, D-mannose, L-galactose, and L-galactono-1,4-lactone, respectively. Data are means ± S.E. of three measurements of each sample. Note the ordinate differential scale representing AsA and DHA contents.

Effect of various possible precursors on AsA and DHA contents Various compounds (25 mM) were tested as possible precursors of AsA biosynthesis. Compounds were added to the SH basal medium supplemented with 20 g/l sucrose. Of the compounds tested, L-galactose (L-Ga) and L-galactono-1,4-lactone (L-Gl) were found effective for the biosynthesis of AsA, whereas sucrose, glucose, and fructose had no effect on AsA content (Fig. 2A). L-Ga markedly enhanced AsA biosynthesis by ca. 6.6 and 5.4 times compared to the control when added at 10 and 17 DAS, respectively, equivalent to the exponential growth stage. L-Gl also significantly increased AsA biosynthesis by ca. 7.5 times when added at 10 DAS. D-Mannose levels increased by ca. 1.6 times when added at 10 DAS, but had otherwise no effect on the level of AsA. DHA content was significantly increased by L-Ga and L-Gl in a similar manner to AsA biosynthesis (Fig. 2B). The absolute content of DHA was ca. 10 times higher than that of AsA regardless of the cell growth stages and kinds of precursors added.

Discussion

AsA has important antioxidant and metabolic functions in both plants and animals. However, humans and a few other animal species have lost the capacity to synthesize this compound (Nishikimi *et al.*, 1994). Thus, plant-derived AsA is the major source of AsA in the human diet. The biosynthetic pathway of AsA in plants has not been well established, even though it is found in millimolar concentrations in plants (Smirnoff, 1996). Two possible pathways for AsA biosynthesis in plants have been suggested (Smirnoff and Pallanca, 1996). The first, known as the inversion pathway, suggests that the immediate

precursor to AsA is L-galactono-1,4-lactone (L-Gl) (Isherwood and Mapson, 1962). The second, a non-inversion pathway, involving the oxidation of glucose at C2 to produce the unusual osone, D-glucosone, has been proposed based on labeling patterns (Loewus *et al.*, 1990; Saito, 1990). In this study, the first inversion pathway of AsA was evaluated in cell cultures of *S. baicalensis* by the feeding of various precursors such as L-Ga and L-Gl.

Plant cell cultures have some advantages in the study of biosynthesis and metabolism of AsA compared to the whole plants. In a previous study, we investigated the AsA content of various plant cell lines and the changes in AsA content during callus cultures of *S. baicalensis*, showing that AsA biosynthesis depends on the concentrations of sucrose, a precursor of AsA, in the medium (Ahn *et al.*, 1998). In this study, the changes in AsA contents and its biosynthetic and metabolically-related enzymes in suspension cultures of *S. baicalensis* were investigated. AsA content slowly increased to 19 DAS, reached a maximum at 21 DAS, and then rapidly decreased with further culturing (Fig. 1A). The sharp decrease of AsA content at 21 DAS may be due to the depletion of carbon sources in the medium, since AsA is biosynthesized from glucose as a starting precursor. The changes in AsA content of suspension cultures showed a similar pattern to those in callus cultures reported previously (Ahn *et al.*, 1998). The high levels of AsA and APX in the early and late growth stages (Fig. 1B) indicate that AsA and APX may contribute as antioxidants to the protection from oxidative stresses derived from subcultures, medium depletion, and cell aging. The levels of the three enzymes investigated during cell growth well reflected the level of AsA as a product or substrate. High POD activity in early and late growth stages has been found in suspension cultures of sweet potato (Kwak *et al.*, 1995).

The feeding experiments using possible precursors indicate that L-Ga and L-Gl are involved in the biosynthesis of AsA in cell cultures of *S. baicalensis*. L-Ga is the most efficient precursor for AsA biosynthesis. These results using cultured cells correlate well with feeding experiments using labeled compounds in *Arabidopsis thaliana* leaves and embryonic axes of germinating pea seedlings (Wheeler *et al.*, 1998). Thus, the cell cultures of *S. baicalensis* should be useful for the isolation of AsA biosynthetic and metabolically-related enzymes and genes for the metabolic engineering of AsA in plants.

In the metabolism of AsA, APX participates in the removal of hydrogen peroxide. The resulting primary oxidation product, a monodehydroascorbate (MDA) radical, is reduced by NAD(P)H-dependent MDA reductase to AsA. Any MDA escaping reduction disproportionates to form AsA and dehydroascorbate (DHA). DHA is directly oxidized by ASO, is unstable at physiological pH, and is reduced to AsA by glutathione (GSH), the reaction being catalyzed by DHA reductase. DHA is also nonenzymatically delactonized to 2,3-diketo-L-gulonic acid, which then produces various breakdown products. Interestingly, DHA in an oxidized form composed more than 90% of the total AsA content in suspension cultures of *S. baicalensis*, even though the ratio of reduced form to oxidized form slightly differed with the cell growth stage (Fig. 2). In the leaves of *S. baicalensis*, a high ratio of DHA to AsA was also observed (unpublished data). In the AsA-GSH cycle in plants (Asada, 1994), reduced AsA in the total AsA pool is known to predominate, maintaining AsA in its reduced form under normal circumstances (Conklin *et al.*, 1996; Smirnoff and Pallanca, 1996). To understand the high ratio of DHA in cultures of *S. baicalensis*, the contents of reduced and oxidized AsA should be analyzed in cultured cells and plant tissues of various plant species. The levels of various enzymes in relation to the AsA-GSH pathway are currently being studied to understand the metabolism and turnover of AsA in the cell culture system of *S. baicalensis*. The GSH content also remains to be determined for understanding the metabolism of AsA in plant cells. Improved understanding of AsA biosynthesis and metabolism in plants should lead to the manipulation of AsA contents with potential benefits for human nutrition and plant resistance to oxidative stress.

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