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Free Radical Scavenging Activity and Ascorbate Content in Various Plant Cell Lines

AHN, Young-Ock[1,3] · CHOI, Yong-Hwa[1] · KWON, Seok-Yoon[1] · LEE, Haeng-Soon[1]
KIM, Suk-Won[2] · PARK, Il-Hyun[3] · KWAK, Sang-Soo[1]*

[1]Plant Biochemistry Research Unit, [2]Genetic Resources Center, Korea Research Institute of Bioscience and Biotechnology, P.O.BOX 115, Yusong, Taejon, 305-600, Korea: and [3]Department of Biochemistry, Chungnam National University, Yusong, Taejon, 305-764, Korea. *Corresponding author

다양한 식물배양세포주에서 자유라디칼 포착활성과 Ascorbate 함량

안영옥[1,3] · 최용화[1] · 권석윤[1] · 이행순[1] · 김석원[2] · 박일현[3] · 곽상수[1]*
[1]생명공학연구소 식물생화학 Research Unit, [2]유전자원센터, [3]충남대학교 자연과학대학 생화학과

We investigated the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity in methanol extracts of 64 cultured cell lines, which were derived from various plant species, and the ascorbate content in cell lines, which showed a high radical scavenging activity. Thirteen cell lines revealed the antioxidative activity (IC_{50}) by methanol extracts of less than 50 mg in cell fresh wt. Of them, six cell lines showed the same R_f value as ascorbate on the DPPH sprayed silica gel TLC. The ascorbate content in cell lines of *Rosa multiflora*, *Scutellaria baicalensis*, and *Achyranthes japonica* showed 48.5, 30.3, and 16.8 μg per g cell fresh wt by HPLC analysis, respectively. In callus cultures of *S. baicalensis*, the concentration of ascorbate reached a maximun (39±3.4 μg/g cell fresh wt) on 30 days after subculture, which corresponded to the stationary growth phase, and subsequently decreased by successive culturing.

Key words: *Scutellaria baicalensis, Rosa multiflora, Achyranthes japonica*, plant cultured cells, DPPH free radical

Oxidative stress by reactive oxygen species (ROS) is a major damaging factor in plants exposed to environmental stress. Aerobic organisms including plants are constantly exposed to the toxic effects of ROS such as superoxide, hydrogen peroxide, and hydroxyl radical. Plants have developed an antioxidant defense system to cope with these ROS. Cellular protective mechanisms against these ROS consist of ROS-scavenging enzymes: i.e., superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT), and low molecular weight antioxidants: i.e., ascorbate (L-ascorbic acid, vitamin C), glutathione, and α-tocopherol (vitamin E) (Alscher and Hess, 1995).

Ascorbate is found in millimolar concentrations in plants, but little is known about its biosynthesis or the role in adaptation of plant against environmental stress. Recently, Conklin et al. (1996) reported that the ozone-sensitive mutant of *Arabidopsis thaliana*, *sozl*, accumulated only 30% of the normal ascorbate concentration. The *sozl* is hypersensitive to both sulfur dioxide and ultraviolet-B radiation and has an altered response to pathogen infection, which implicated that ascorbate might play an important role in defense against various biotic and abiotic environmental stresses (Last et al., 1997).

On the other hand, plant cell cultures are very important not only for supporting the field of plant biotechnology but also for the mass production of useful compounds or mass propagation of useful plants. Cultured plant cells are considered to be grown under high oxidative stress conditions, which suggests that plant cell cultures are an efficient system for study of antioxidative mechansim and production of useful

antioxidants. In previous papers, 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). Possible mass production of POD and physiological role of each isoenzyme in terms of cellular adaptation against environmental stresses have been investigated in sweet potato cell line (Kim et al., 1994: Kwak et al., 1995, 1996: Huh et al., 1997, 1998). In the present paper, we described the activity of low molecular weight antioxidants in various plant cell lines using the 1,1-diphenyl-2-picrylhydrazyl (DPPH), which is a free radical showing a characteristic absorption at 517 nm (purple), and the changes in ascorbate content during callus cultures of *Scutellaria baicalensis*.

MATERIALS AND METHODS

Plant materials and cell cultures

Plant cell lines in maintaining at Plant Research Laboratories and Korean Collection of Type Cultures (KCTC), Korea Research Institute of Bioscience and Biotechnology, were used (Liu and Yang, 1991). Two hundred mg (fresh wt) of the calli subcultured at 25-day intervals was inoculated into medium supplemented with 30 g/L sucrose and 0.4% Gelrite at 25°C in the dark. The cells at stationary growth stage were harvested for antioxidant activity.

DPPH free radical scavenging activity

The antioxidative activity using a 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical was determined according to the method of Xiong et al. (1996) with a slight modification. The cells (200 mg fresh wt) were homogenized in 0.5 mL methanol on ice for 1 min and centrifuged at 10000 × g for 10 min. The resulting supernatant was used as a crude extract for a DPPH free radical scavenging activity. One mL of 0.15 mM DPPH in ethanol was added to sample solution containing 4 mL of methanol and allowed to react for 30 min at room temp. After finishing reaction, the optical density was measured at 517 nm. For UV spectrophotometer assay, ethanol was used as a blank instead of DPPH and methanol was used as a control instead of the sample solution. The IC_{50} values were calculated from the regression lines where the abscissa represented the concentration of tested compounds and the ordinate represented the average percent deduction of DPPH radical from three separate tests. The detection of antioxidative components on TLC were done by spraying with a DPPH solution according to the Takao et al. (1994). The crude extract with 62.5 mM metaphosphoric acid (MPA) was developed onto the TLC (silica gel $60F_{254}$) using methanol and acetone (8:3) and the detection of antioxidative compounds including ascorbate were done by spraying with DPPH solution (80 μg/mL). The R_f value of an authentic ascorbate was 0.5 in this condition.

Ascorbate analysis by HPLC

HPLC analysis for ascorbate was conducted according to Graham and Annette (1992). The crude extract was filtered through a 0.5 μm FH-type Millipore filter. This sample was loaded onto a column of Bio-rad aminex HPX-87H (300 x 7.8 mm). The 4.5 mM sulfuric acid was eluted at a flow rate of 0.5 mL/min, and the optical density of ascorbate was measured at 245 nm. The R_t of an authentic ascorbate was 12.3 min. The quantitative analysis was carried out by comparing the peak areas of the samples with that of the authentic ascorbate.

RESULTS AND DISCUSSION

The antioxidative activity of the plant cell lines against a DPPH free radical varied in cell lines (Table 1), which reflects their diverse antioxidative mechanism, even though they were induced and grown under different culture conditions. Of the 64 cell lines tested, 13 cell lines showed a high activity below 50 mg fresh wt required for 50% reduction of a DPPH free radical after reaction for 30 min, whereas 51 cell lines showed a relatively low activity. In the antioxidative components test using the selected 13 cell lines, diverse DPPH positive reaction spots were observed on TLC depending on the cell lines (data not shown). Six cell lines of *Achyranthes japonica, Leonurus sibricus, Lithospermum erythrorhizon, Magnolia salicifolia, Rosa multiflora, and Scutellaria baicalensis* had strong positive spots near the R_f value of an authentic ascorbate. However, other 7 cell lines showed no DPPH positive reaction spots near the R_f value of an authentic ascorbate, which suggests that the major antioxidative components in these cell lines are derived from different antioxidants aside from ascorbate.

Ascorbate content was determined by HPLC in the six cell lines which showed a similar R_f value with ascorbate on

Table 1. Antioxidative activity of plant cell lines against a DPPH radical.

Plant species (KCTC PCL No)[a]	Culture medium[b]	IC$_{50}$[c] (mg cell fresh wt)
Achyranthes japonica (10002)	MS2N1B	7
Leonurus sibricus (10096)	MS1D	9
Lithospermum erythrorhizon (10099)	MSBM	18
Magnolia salicifolia (10103)	MS1D	27
Nicotiana tabacum (10112)	MS2N1B	42
Oenanthe javanica (10114)	V	32
Oenothera odorata (10116)	MS2N1B	36
Panax ginseng (10122)	MSBM	5
Phytolacca esculenta (10134)	MS1D	33
Raphanus sativus (10147)	MS1D	15
Rosa multiflora (10151)	SH	14
Rosa multiflora (10152)	SH	3
Scutellaria baicalensis (10159)	SH	25
Other 51 cell lines		> 50
Ascorbate		0.35 μg

[a]KCTC PLC : Korean Collection for Type Cultures, Plant Cell Lines (Liu and Yang, 1991).

[b]Thirty g/L sucrose and 0.4% Gelrite were added to all media. The pH of all media was adjusted to 5.8 before autoclaving. MS2N1B : Murashige and Skoog (1962) medium containing 2 mg/L NAA and 1 mg/L BA: MS1D : MS medium containing 1 mg/L 2,4-D: MSBM : MS basal medium: V : B5 medium (Gamborg et al., 1968) containing 1.5 mg/L 2,4-D, 0.1 mg/L IAA and 0.2 mg/L kinetin: SH : Schenk and Hildebrandt (1972) medium containing 2 mg/L p-PCA, 0.5 mg/L 2,4-D and 0.1 mg/L kinetin.

[c]Amount required for 50% reduction of a DPPH free radical after reaction for 30 min.

Table 2. Ascorbate content was determined by HPLC in six cell lines which showed a similar *Rf* value with ascorbate on TLC after spraying with a DPPH reagent.

Plants species (KCTC PCL No)	Ascorbate content(μg/g cell fresh wt)
Achyranthes japonica (10002)	16.8
Leonurus sibricus (10096)	ND[a]
Lithospermum erythrorhizon (10099)	ND
Magnolia salicifolia (10103)	ND
Rosa multiflora (10152)	48.5
Scutellaria baicalensis (10159)	30.3

[a]ND : not detected by HPLC.

TLC. Three of them showed the peaks which were well matched with an authentic ascorbate by cochromatography (Figure 1). Ascorbate content in three cell lines (i.e., *R. multiflora*, *S. baicalensis*, and *A. japonica*) was 48.5, 30.3, and 16.8 μg per g cell fresh wt, respectively. However, ascorbate content was not observed in other cell lines (i.e., *L. sibricus*, *L. erythrorhizon*, *and M. salicifolia*), which suggested that either there was no production of ascorbate or too little ascorbate to be detected by HPLC (Table 2).

The radical scavenging activity of methanol extracts in cell lines is well correlated with that of metaphosphoric acid (MPA) extracts. Thus, the DPPH radical scavenging assay is a good system to evaluate the antioxidative activity as well

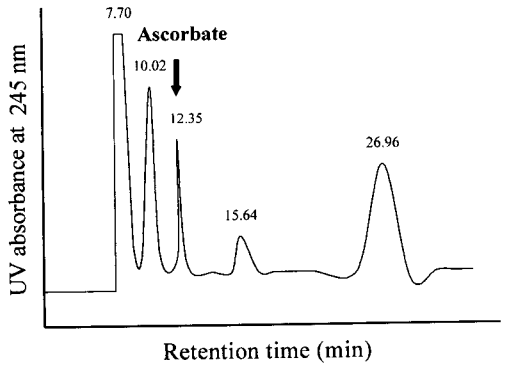

Figure 1. HPLC analysis of ascorbate in callus of *Scutellaria baicalensis*. The sample was loaded onto a column of Bio-rad aminex HPX-87H (300 x 7.8 mm). The 4.5 mM sulfuric acid was eluted at a flow rate of 0.5 mL/min.

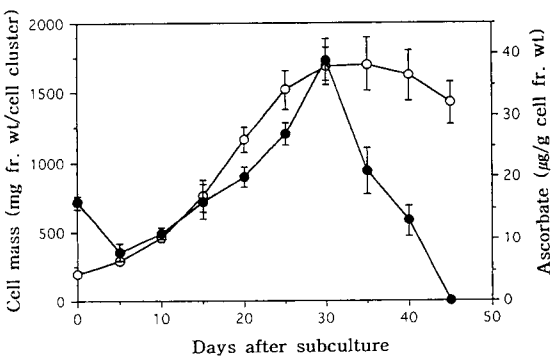

Figure 2. Changes in ascorbate content during callus cultures of *Scutellaria baicalensis* in SH basal medium supplemented with 30 g/L sucrose and 0.4% Gelrite. The data are means±S.E. of three replicates. ○ - ○ : cell mass (mg fresh wt per cell cluster): ● - ● : ascorbate content (μg/g cell fresh wt).

as to screen the cell lines producing ascorbate. For the investigation of relationship between cell growth and ascorbate content, we measured the changes in ascorbate content during callus cultures of *S. baicalensis* which showed a high concentration of ascorbate (Table 2) and a good cell growth. The cell mass of *S. baicalensis* was maximum on 30 days after subculture (DAS) showing a typical sigmoid growth curve (Figure 2). The ascorbate content is well correlated with the cell mass until the stationary growth phase and subsequently decreased by successive culturing. The maximum content of ascorbate (39±3.4 μg/g cell fresh wt) was observed on 30 DAS. No ascorbate production was observed at the end of cell growth (45 DAS). In our previous studies, the activity of two antioxidant enzymes of POD and SOD

showed the lowest level at the exponential growth stage and the highest level at the end of cell cultures of sweet potato (Kwak et al., 1995), and tomato (You et al., 1997). The observed results in the present study suggest that the ascorbate content in callus cultures of *S. baicalensis* is closely associated with cell growth and that the ascorbate biosynthesis depends on the concentrations of sucrose, the precursor of L-ascorbate, in the medium.

The biosynthetic pathway of ascorbate in plants has not been well established. The terminal step in the biosynthesis of L-ascorbate has been shown to be oxidation of L-galactono-1,4-lactone by an enzyme present in mitochondria preparation (Mapson et al., 1954). However, biosynthetic function of L-galactono-1,4-lactone dehydrogenase (GLDase) converting L-galactono-1,4-lactone to L-ascorbate is poorly characterized in plants. The cells of the late exponential growth phase in *S. baicalensis* is good material for the study of ascorbate biosynthesis including the purification of GLDase. Recently, GLDase was purified from plant materials such as potato roots (Oba et al., 1994), sweet potato roots (Oba et al., 1995), and cauliflower florets (Ostergaard et al., 1997). A cDNA coding for GLDase was isolated and characterized from cauliflower (Ostergaard et al., 1997). However, there is no report on the ascorbate content and ascorbate-related enzymes in cultured plants cells, even though plant cell cultures have some advantages in the study of ascorbate biosynthesis. The cell lines producing ascorbate in this study may be useful for the study of ascorbate biosynthesis and the understaning of antioxidative mechanism in plant cells. The changes in the level of ascorbate and ascorbate-related enzymes in suspension cultures of *S. baicalensis* are under investigation.

적 요

다양한 유도조건에서 확립한 64종의 식물배양세포주의 MPA추출물을 대상으로 DPPH 라디칼을 이용하여 항산화 활성을 조사하고 활성이 높은 세포주를 대상으로 HPLC로 ascorbate 함량을 분석하였다. 13종의 세포주는 생체중 50 mg 이하에서 DPPH 라디칼의 자연산화를 억제하는 높은 항산화활성을 나타내었다. 이중 6종의 세포주의 MPA추출물을 실리카겔 TLC에 전개하여 DPPH용액을 분무한 결과, ascorbate와 같은 R_f 위치에서 DPPH 양성반응을 나타내는 화합물이 존재하였다. DPPH 양성반응을 나타낸 덩굴장미(*Rosa multiflora*), 황금(*Scutellaria baicalensis*), 쇠무릎(*Achyranthes japonica*) 세포주의 ascorbate 함량(㎍/g cell fresh wt)은 각각 48.5, 30.3, 16.8였다. 황금 캘러스 배양에서 ascorbate의 함량은 세포생장에 비례하여 정지기까지 증가한 후 계속적인 생장에 따라 급격히 감소하였다.

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