The Production of Anti-cancer Substances by *in vitro* Grown Cultures of *Panax ginseng* C.A. Meyer

Deok-Chun Yang, Kyung-Hwa Choi[1], Yong-Hae Kim, Eui-Soo Yoon[2], Tae-Jin Kang[3] and Kwang-Tae Choi

Korea Ginseng & Tobacco Research Institute, Taejon, 305-345, Korea;
[1]Plant Tissue Culture Research Unit, KRIBB, Taejon, 305-600, Korea
[2]Department of Biology, Kongju National University, Kongju 314-701, Korea
[3]Neurobiotechnology Center, Ohio State University, Columbus, OH 43210, USA

ABSTRACT

Ginseng(*Panax ginseng* C.A. Meyer) is important medicinal plant but requires 4-year cultivation for root harvest because of slow growth. In contrast, ginseng callus and hairy roots grow vigorously and may produce the same or more biologically active compounds for human health than natural ginseng roots. Therefore, ginseng callus and hairy roots can be used for commercial purposes. Polyacetylene, one of anti-cancer compounds in ginseng, was not detected in the callus cultured on the medium containing 2,4-D, but cells derived from the callus growth was excellent. The ginseng calli cultured on the medium containing 2mg/l CPA and 0.05mg/l BA was grown vigorously and produced panaxydol, one of ginseng polyacetylene. The biosynthesis of polyacetylene in callus was not affected by addition of NAA and sucrose in media. The SH medium was better than the MS medium for ginseng callus growth and biosynthesis of panaxydol. Another ginseng anti-cancer compounds, ginsenoside-Rg_3, Rh_1 and Rh_2 were detected in ginseng hairy roots by heat treatment. Those of *Panax ginseng* were obtained after root disks of three-year old roots were infected with *Agrobacterium rhizogenes* R1000 A_4T in dark condition after one month of culture. The optimum growth of hairy roots was achieved in the culture of 1/2 MS liquid medium in dark(22℃) under 60 rpm gyratory shaking. Hairy roots grew well in 5 *l* Erlenmeyer flasks, 1 *l* roller drums, 10 *l* jar-fermenters, and especially in 20 *l* air-lift culture vessels. All heat treatments had remarkably different ginsenoside contents. Eleven ginsenosides were determined in heat treatment, eight in freeze dried hairy roots. Contents of ginsenoside-Rb1, Rb2, Rc, Rd, Re, Rf, and Rg_1 tested in all heat treatments were less than those of freeze dried hairy roots. Contents of ginsenoside-Rg_2 in heat treatment for 1 hour at

105° C was 4.92mg/g dry wt, 3.9 times higher than 1.27 mg/g dry wt of freeze dried hairy roots. The optimum condition of heat treatment for the production of ginsenoside-Rg_3 and Rh1 was 2 hours at 105° C, and ginsenoside content was 2.58mg/g dry wt and 3.62mg/g dry wt, respectively. The production of ginsenoside-Rh_2 was the highest in heat treatment for 2 hours at 105° C among treatments examined, and ginsenoside-Rh_2 content was 1.08mg/g dry wt.

Key words : Heat treatment, ginseng, polyacetylene, ginsenoside

INTRODUCTION

Korean ginseng(*Panax ginseng* C.A. Meyer) is representative medicinal plants in Korea and have various pharmacological effects in human bodies. Lately, the ginseng is manufactured into various kinds of health foods, thus the demand is increased gradually. In addition, the ginseng saponins can be used for medicinal purpose. Recently, the necessity on the developments and applications of medicinal plant resources were activated concomitantly with the reinforcement of international trends on the patent law. The plant tissue and cell culture methods were applied to the mass production of medicinal components including anticancer substances(Han and Kim, 1983; Hong *et al.*, 1979; Hwang and Oh, 1984). Especially, it is reported that ginseng shows the anticancer effects, and that several kinds of polyacetylene compounds including panaxydol and Panaxatriol(Shin *et al.*, 1983), and ginsenoside compounds Rg_3(Mochizuki *et al.*, 1995a, 1995b), Rh1(Odashima *et al.*, 1979; Lee *et al.*, 1996) and Rh2(Tode *et al.*, 1993; Kikuchi *et al.*, 1991) in ginseng showed the toxicity on the leukemia cells. It is suggested that the attempts of mass production of useful non-side effective anticancer agent are important. However, the improvement of ginseng productivity is extremely restricted by the physiological characteristics of this plant, i.e. low growth rate as a perennial semi-shade plant and high susceptibility to disease and environmental stresses. Therefore, new biotechnological techniques such as tissue and cell cultures(Hagimori *et al.*,1982; Yang *et al.*, 1998) should be applied for mass production of medicinal and biologically active components. Ginseng callus culture has been established from somatic tissues of *Panax ginseng* C.A. Meyer. Furuya et al(1983) studied the large-scale culture of *Panax ginseng*. As a result, callus tissues and redifferentiated roots were able to accumulate saponins and sapogenins

which have been known as the metabolites in the intact ginseng plant. Production of ginseng saponin(ginsenosides) from hairy roots induced by *Agrobacterium*-mediated transformation can be used for efficient method since the hairy roots are genetically stable and show active growth potential in hormone-free medium(Yang *et al.*, 1998).

The objectives of this study are (1) to promote the synthesis of biologically active compound(polyacetylene) by the treatments of plant hormone in ginseng tissue culture, (2) to produce ginsenoside-Rg_3, Rh_1 and Rh_2 from ginseng hairy roots by heat treatment, and to accomplish the mass production of bioactive metabolite for commercial application.

MATERIALS AND METHODS

[Experiment 1]

Analysis and culture conditions for biosynthesis of polyacetylene from ginseng cultured Cells

Effect of NAA and BA for ginseng callus growth and biosynthesis of polyacetylene

To investigate BA effect affecting biosynthesis of polyacetylene and callus growth of ginseng. 2mg/l of ρ-chlorophenoxyacetic acid(CPA) was added to Murashige and Skoog(MS, 1962) medium. After adding CPA, the concentration of 6-benzylaminopurine(BA) was adjusted to 0, 0.05, 0.1, 0.5, 1.0, 2.0, 5.0mg/l, and then callus was inoculated. Growth rate and the amount of polyacetylene were tested by TLC after 30days of culture. In addition, only 0, 0.1, 0.5, 1.0, 2.0, 5.0mg/l of NAA was added and investigated for the effect on Naphthalene acetic acid(NAA) in the cultural condition at $25 \pm 2^{\circ}$C with a photoperiod of 16 hours daylight at an weak light intensity($3\mu moles/m^2/s$).

Effect of sucrose and media for ginseng callus growth and biosynthesis of polyacetylene

To investigate the effect of sucrose, 10, 20, 30, 40, 50g/l of sucrose with CPA 2mg/l was used for culture. In addition, the effect of callus growth and different medium kinds was tested by adding CPA 2mg/l to Schenk and Hildebrandt(SH) and MS medium.

Analysis of polyacetylene from ginseng callus by TLC technique

The existence of polyacetylene was tested by thin layer chromatogram(TLC) method for applying the confirmed callus to gas chromatogram(GC) after swiftly confirming the existence of polyacetylene from the callus of ginseng. Freeze dried 0.5g of ginseng callus was extracted under the solvent mixed with 4:1 of petroleum ethel and ethel for 15 hours and again concentrated. The concentrated extract was diluted to 500μl by the same solvent, and 50μl of it was applied to TLC. For TLC development solvent, 8:2 of petroleum ethel and ethel was used and colored by anisaldehyde sulfuric acid.

[Experiment 2]

Mass production of ginsenoside-Rg3, Rh1 and Rh2 from ginseng hairy roots by heat treatment

Induction of hairy roots and culture

Ginseng(*Panax ginseng* C.A. Meyer) roots of 3-years old were surface-sterilized by NaOCl and pieced to root disks. The root disks were co-cultured with *A. rhizogenes* R1000 or *A. tumefaciens* A4T, then transferred to hormone-free MS medium with 500μg/al carbenicillin at 25° C in dark condition. For the subculture of cell line on hairy root, 0.5g of fresh weight was used to MS(40ml/100ml flask) liquid medium supplemented with vitamin of Gamborg B5(Gamborg *et al.*, 1968) liquid medium and 3% of sucrose in the interval of 3 weeks. Hairy root cultured in the dark condition at 23° C for 4 weeks in the 100ml erlenmeyer flask containing 40ml MS/B5 liquid medium with 1g of fresh weight was used for heat treatment.

Heat treatment of hairy roots

For heat treatment of hairy roots, hairy roots collected after culture were washed twice by distilled water followed by removing water from hairy roots. The samples were tested by different temperature and time by using steam sterilizer. The temperature and time for heat treatment was 1) 90° C for 30 and 60min, 2) 105° C for 30, 60, 120, 180min, 3) 121° C for 30 and 120min. The hairy roots treated by heat was dried by dryer which is maintained constantly at 60° C.

Isolation and analysis of ginsenoside

Ginsenoside was extracted by n-BuOH extraction method. One g of dried powder sample was

used to extract by using 80% of MeOH in the water bath at 60° C, dried, extracted by ether, and de-fated. They were extracted by n-BuOH and washed by distilled water followed by drying n-BuOH layer, and they were used for analysis sample of TLC and HPLC. To analyse TLC of ginsenoside extracted, 5μl of crude saponin was spotted to the TLC plate(silica gel 60 F_{254}, Merck) and developed to low layer of $CHCl_3$:MeOH:H_2O (65:35:10, v/v). Color was expressed in the dryer at 105oC after spraying 30% H_2SO_4. Quantitative analysis of ginsenoside was performed using detector of refractive index(RI) (Waters R401). LiChrospher-NH2 column(Merk Co., 10μm, 0.46cm I.D×25cm), acetonitrile/H_2O/n-Butanol(80:20:10, v/v) and acetonitrile/ H_2O (90:10, v/v) for mobile phase were used. U6K of Waters company was used for pump. Flow rate was calculated by peak height of ginsenoside content, which was compared with standard of peak on chromatogram under the condition of 0.3ml/min과 2.0ml/min.

RESULTS AND DISCUSSION

[Experiment 1]

Analysis and culture conditions for biosynthesis of polyacetylene from ginseng cultured Cells

Effect of NAA and BA for ginseng callus growth and biosynthesis of polyacetylene

Ginseng grew very well in the medium supplemented with 2,4-D or CPA, but no polyacetylene was detected in that medium(Table 1). Therefore, the best CPA concentration(2mg/ml) for the growth of ginseng callus and different BA and NAA concentration was applied to the culture. To determine NAA effect affecting the growth of ginseng callus and polyacetylene, different concentration of NAA treatment was used for culture. As a result, the amount of callus growth rate was gradually decreased as NAA concentration increases, and polyacetylene was not detected in all the treatments of both panaxynol and panaxydol.

Table 1. Effect of NAA on the growth and production of polyacetylene in ginseng callus* on the MS medium with 2mg/l CPA

Conc. of NAA(mg/l)	Fresh weight(mg/flask)	Panaxynol	Panaxydol
0	11.754±0.438	-**	-
0.1	11.666±0.509	-	-
0.5	11.575±0.250	-	-
1.0	11.561±0.939	-	-
2.0	10.927±1.132	-	-
5.0	10.827±1.001	-	-
2,4-D 3mg/l	12.345±0.214	-	-
Fresh ginseng Root		++++	+++++

* Callus was cultured on the basal medium with 2mg/l CPA for 30 days

** - : Not detected, +++++ : excellent.

To investigate the effect of BA, growth rate according to different BA concentration and polyacetylene formation was investigated in the medium containing 2mg/l of CPA. As a result, growth rate was the best in the treatment of 0.05mg/l BA, and fresh rate of callus was decreased as the concentration was getting increased. More than 2mg/l of BA showed 5.642 g/flask of fresh weight, which showed more than half of decreased fresh weight rate(Table 2). In addition, panaxydol of polyacetylene was detected with BA treatment, which was not formed in the treatment of one treatment of 2,4-D or CPA. However, panaxydol content was not increased even though BA concentration increased. The panaxydol content detected was also very little compared to ginseng roots(Table 2).

Table 2. Effects of BA on the growth and production of polyacetylene in ginseng callus* on the MS medium with 2mg/l CPA

Conc. of BA(mg/l)	Fresh weight(mg/flask)	Panaxynol	Panaxydol
0	9.208±0.950	-**	-
0.05	11.032±0.448	-	+
0.1	8.162±0.458	-	+
0.5	8.263±0.650	-	+
1.0	8.256±0.954	-	+
2.0	5.642±0.406	-	+
5.0	5.774±0.503	-	+
Fresh ginseng root		++++	+++++

* Callus was cultured on the basal medium with 2mg/l CPA for 30days

** - : Not detected, + : detected, +++++ : excellent.

Effect of sucrose and media for ginseng callus growth and biosynthesis of polyacetylene

Sucrose concentration was treated by 10-50mg/l to investigate the effect of sucrose affecting the production of ginseng callus and polyacetylene. As a result, growth was the best in the concentration of 30-40mg/l of sucrose treatment, and half of growth was decreased in the concentration below 20mg/l, and growth was also decreased in the concentration of 50mg/l of sucrose(Table 3). Polyacetylene was not at all detected in the all treatment regardless of sucrose concentration.

Table 3. Effect of sucrose on the growth and production of polyacetylene in ginseng callus*

Conc. of Sucrose(mg/l)	Fresh weight(mg/flask)	Panaxynol	Panaxydol
10	5.147±0.328	-**	-
20	6.881±0.921	-	-
30	10.403±0.921	-	-
40	10.812±0.633	-	-
50	9.978±0.500	-	-
Fresh ginseng root		+++	++++

* Callus was cultured on the basal medium with 2mg/l CPA for 30days

** - : Not detected, +++++ : excellent.

The optimal medium for callus growth and polyacetylene formation was tested. As the result, SH medium was better than MS medium in terms of the growth of ginseng callus. Especially, panaxydol was a little detected in the SH medium.

Table 4. Effect of basal media on the growth of callus and production of polyacetylene in ginseng callus*

Media	Fresh weight(g/flask) of callus cultured for		Polyacetylene	
	15days	30days	Panaxynol	Panaxydol
Schenk and Hildebrandt	3.43±0.26	8.72±0.49	-	+
Murashige and Skoog	2.08±0.09	7.64±0.35	-	-
Fresh ginseng root			++++	+++++

* Callus was cultured on the basal medium with 2mg/l CPA for 30days

** - : Not detected, + : detected, +++++ : excellent.

[Experiment 2]

Mass production of ginsenoside-Rg_3, Rh_1, and Rh_2 from ginseng hairy roots by heating treatment

Induction and culture of hairy roots

After co-culture of root disks with *Agrobacterium rhizogenes* R1000 or *A. tumefaciens* A_4T, hairy roots were induced. About 3 hundred lines of hairy roots were selected and maintained by root tip culture. All the hairy roots were identified to transformed roots by PCR analysis. Hairy roots actively grew in 1/2MS liquid medium at 22° C in the dark condition. The appropriate initial inoculation weight of hairy roots were 1g in 100ml Erlenmeyer flask.

Effect of culture type vessels on the growth of hairy roots

Hairy roots were grew well in the various culture system. When the 50g of hairy roots were cultured in the 30L air lift culture vessel with 20L culture medium, about 1,500g/FW of hairy roots were gained after 2 months of culture(Table 5).

Table 5. Growth of ginseng hairy roots on the various culture vessels

Vessel	Media volume	Initial inoculum (g fresh wt)	Growth (g fresh wt)	Air supply
100 ml flask	40 ml	1	8	shaking
250 ml flask	100 ml	4	30	shaking
2.5L air lift	1 L	10	200	air lift
5 L air lift	2 L	20	450	air lift
10 L jar fermenter	5 L	20	600	air lift
30L air lift	20 L	50	1500	air lift

Heat treatment of hairy root

For the method of mass production on ginsenoside-Rh_2, the typical ginsenoside of red ginseng, and ginsenoside-Rh_1 and Rg_3 which is found mainly in red ginseng rather than white ginseng, heat treatment was used by different temperature and time. As a result, distinct ginsenoside content was found according to different treatments(Table 6). The content of ginsenoside-Rb_1, Rb_2, Rc, Rd, Re, Rf, Rg_1 was decreased compared to freeze dried treatment(Table 6). The treatment showing the highest decrease of 7 ginsenoside content was the treatment at

105° C for 3 hours and 121° C for 1 hour of heat treatment. Hydrolysis of 7 ginsenoside was higher at 121° C for 1 hour than that at 105° C for 3 hour treatment. Therefore, it was confirmed that high temperature was better. Ginsenoside-Rg_2 content was decreased at 90° C treatment, whereas it was increased at 105° C and 121° C treatment. Especially, 105° C treatment showed increase according to the increase of treatment time, however, it was decreased again in the 3 hour treatment. When it was treated at 105° C for 1 hour, ginsenoside-Rg_2 content was 4.92mg/g dry wt, which was 3.9 times as much as freeze dried treatment, 1.27mg/g dry wt(Table 6). Ginsenoside-Rg_3 is existed little in the white ginseng, but existed much in the red ginseng. The optimal condition for ginsenoside-Rg_3 formation is the heat treatment at 105° C for 2 hours, which showed 2.58gm/g dry wt(Table 6). In addition, ginsenoside-Rh_1 is also contained much in red ginseng. Little content was found from the sample of freeze dried sample of hairy root. The optimal condition for the formation of ginsenoside-Rh1 is the same condition as ginsenoside-Rg_3, 105° C for 2 hours heat treatment, forming 3.62mg/g dry wt(Table 6). Ginsenoside-Rh_2, the typical ginsenoside of red ginseng, is formed in the process of red ginseng production. The optimal condition of ginsenosides-Rh_2 production is the heat treatment at 105° C for 2 hours, showing 1.08mg/dry wt. As the above result, the optimal condition for the typical components of red ginseng, ginsenoside-Rg3, Rh1, Rh2 is heat treatment at 105° C for 2 hours(Table 6).

The rate of panaxatriol-ginsenoside(PT) to panaxadiol ginsenoside(PD) was 0.97 in the freeze dried treatment, whereas 0.77 in the heat treatment at 105° C for 2 hours showing high rate of PT.

The reason for decrease of rate on PT to PD(PD/PT) is because of more production of ginsenoside-Rg_2, Rh1 than ginsenoside-Rh_2, Rg_3. Ginsenoside-Rh_2, Rg_3 content of freeze dried treatment is 0mg/g dry wt, whereas 3.66mg/g dry wt was found in the treatment of 105° C for 2 hous, which shows 3.66mg increase. The content of ginsenoside-Rg_2, Rh1 is because of high increase, 7.27mg, compared to freeze dried treatment. The condition to covert to typical ginsenoside-Rg_3, Rh_1, Rh_2 of red ginseng from hairy root of ginseng is the heat treatment at 105° C for 2 hours. It is considered that the change to ginsenoside-Rg_2, Rg_3, Rh_1, Rh_2 during heat treatment process is the result from the hydrolysis of ginsenoside. The saponin of ginseng is triterpenoid of dammarane. It is classified to panaxadiol and panaxatriol according to the number of OH group of aglycone. Ginsenoside is composed of ethel bond of sugar such as glucose, rhamnose, xylose, arabinose to C-3 and C-20 or C-6 and

C-20(Park, 1996). When ginsenoside is hydrolyzed by weak acid such as nitric acid, only glycosidic bond of C-20 is hydrolyzed and prosapogenin is formed. When ginsenoside-Rb_1, -Rb_2, -Rc, -Rd are hydrolyzed, prosapogenin, 20(R&S)-ginsenoside Rg_3, is formed(Shibata *et al.*, 1965). In addition, when PT ginsenoside, ginsenoside-Re, -Rf, -Rg_1, is hydrolyzed, different prosapogenin is formed. In case of ginsenoside Re, it is reported that 20(R&S)-ginsenoside-Rg_2 is formed(Park, 1996). In conclusion, it is considered that the high formation of ginsenoside-Rg_2, Rg_3, Rh_1, Rh_2 according to heat treatment of hairy root is the result of hydrolysis of sugar bound to C-20, and it is assumed that the degree of hydrolysis of each ginsenoside is based on the temperature and time of heat treatment. Therefore, it is considered that the mass production of ginsenoside-Rg_2, Rg_3, Rh_1, Rh_2 will be possible by way of the heat treatment of ginseng hairy root cultured in high amount.

Table 6. Ginsenoside contents according to temperature and time of heat treatment in ginseng hairy roots

Temp (°C) - Time (min)	Ginsenoside contents (mg/g dry wt)														
	Rb_1	Rb_2	Rc	Rd	Re	Rf	Rg_1	Rg_2	Rg_3	Rh_1	Rh_2	PD	PT	PD /PT	Total
FD	5.66	3.96	1.74	1.93	6.83	0.34	5.32	1.27	T	T	0	13.29	13.76	0.97	27.05
	(20.9)*	(14.6)	(6.4)	(7.1)	(25.3)	(13.3)	(19.7)	(4.7)	(0)	(0)	(0)	(49.1)	(50.9)		
90-30	1.92	1.21	0.63	1.19	2.16	0.32	3.14	0.32	0.02	0.12	0.02	4.99	6.06	0.82	11.05
90-60	2.21	1.54	0.74	1.19	2.54	0.31	2.24	0.48	0.04	0.46	0.13	5.85	6.03	0.97	11.88
105-30	2.84	1.98	0.84	1.29	3.21	0.29	3.14	1.27	0.45	0.87	0.24	7.64	8.78	0.87	16.42
105-60	4.54	3.08	1.26	1.62	5.48	0.28	4.98	2.22	0.56	1.81	0.47	11.53	14.77	0.78	26.30
105-120	3.77	2.64	1.05	1.67	3.56	0.25	4.34	4.92	2.58	3.62	1.08	12.79	16.69	0.77	29.48
	(12.8)	(8.9)	(3.6)	(5.7)	(12.1)	(0.9)	(14.7)	(16.7)	(8.8)	(12.3)	(3.7)	(43.4)	(56.6)		
105-180	1.70	1.10	0.42	0.62	1.92	0.23	1.62	2.78	0.42	1.76	0.36	4.62	8.31	0.56	12.93
121-30	2.41	1.32	0.63	0.74	2.86	0.32	2.44	2.06	0.53	1.43	0.45	6.08	9.11	0.67	15.19
121-60	1.41	0.77	0.38	0.49	1.76	0.28	1.62	2.86	0.97	1.85	0.62	4.64	8.37	0.55	13.01

FD: Freeze dried hairy root, T: Trace, PD: Ginsenoside-Rb_1+Rb_2+Rc+Rd+Rh_2+Rg_3,
PT: Ginsenoside-Re+Rf+Rg_1+Rg_2+Rh_1,
* : % / Total ginsenoside

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