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Production of Red Ginseng Specific Ginsenosides (Rg_2, Rg_3, Rh_1 and Rh_2) from *Agrobacterium* - transformed Hairy Roots of *Panax ginseng* by Heat Treatment

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It was reported that Red ginseng contains specific ginsenoside-Rg_2, -Rg_3, -Rh_1 and -Rh_2, which show various pharmacological effects. However, production of these specific ginsenosides from Red ginseng is not commercially applicable because of high cost of the raw material, roots. This work was carried out to examine the production of Red ginseng specific ginsenosides from *Agrobacterium*-transformed hairy roots. Hairy roots were induced from 3 year-old root segments of Korean ginseng (*Panax ginseng* C.A. Meyer) after infection with *Agrobacterium rhizogenes* A4. Among many lines of hairy roots, KGHR-8A was selected. Steam heat treatment of hairy roots was resulted in the changes of ginsenoside composition. Eleven ginsenosides were detected in heat-treated hairy roots but eight in freeze dried hairy roots. In heat treated hairy root, content of ginsenoside-Rb_1, Rb_2, Rc, Rd, Re, Rf, and Rg_1 were decreased compared to those of freeze dried hairy roots. However, heat treatment strongly enhanced the amount of Red ginseng specific ginsenosides (ginsenoside-Rg_2, -Rg_3, -Rh_1 and -Rh_2). Amounts of ginsenoside-Rg_3, -Rh_1 and -Rh_2 in heat-treated hairy roots were 2.58, 3.62 and 1.08 mg/g dry wt, respectively, but these were detected as trace amount in hairy roots without heat treatment. Optimum condition of heat treatment for the production of Red ginseng specific ginsenoside was 2 h at 105°C. This result represents that Red ginseng specific ginsenoside can be produced from hairy roots by steam heat treatment.

Key words: Red ginseng, heat treatment, hairy root, ginsenoside

INTRODUCTION

Korean ginseng (*Panax ginseng* C.A. Meyer) is a perennial herbaceous plant and has been recognized as a miraculous medicinal plant. Various pharmacological effects of Korean ginseng were reported, such as tonic, adaptogenic, antistress, hypothermic, minor hyperglycemic, and anticancer activity [1]. The pharmacologically active main component of ginseng was largely accepted as saponin such as panaxadiol, panaxatriol, and oleanolic acid [2]. More than 20 of ginsenosides was reported in Korean ginseng and each ginsenoside has specific pharmacological effect [1].

Red ginseng is made from steam heat treatment of fresh ginseng roots. The red ginseng has some superior pharmacological effects compared to fresh or dried ginseng roots [3]. The red ginseng contains specific ginsenoside-Rh_1, ginsenoside-Rh_2, 20(S) - ginsenoside Rg_2, 20(S) - ginsenoside Rg_3, and these are not detected or even as trace amount in fresh and dried ginseng roots [4]. Ginsenoside-Rg_3 showed anti-tumor [5], neuroprotective activity [6], and relaxing activity of vascular smooth muscle [7,8]. Ginsenoside-Rh_1 and -Rh_2 showed apoptosis of human hepatpma [9], and anti-tumor [10-13]. These specific ginsenosides of Red ginseng can be used for important medicine. However, commercial use of Red ginseng specific ginsenosides is very difficult because of high price of the root.

Ginseng hairy roots induced by *Agrobacterium rhizogenes*-transformed show vigorous growth and produce equal amounts or more saponins than non-transformed ginseng roots [14], which shows that ginsenoside can be produced from ginseng hairy roots. It has not been reported, so far on the production of Red ginseng specific ginsenosides from ginseng hairy roots.

This paper was carried out to examine the production of Red ginseng specific ginsenosides (Rg and Rh groups) from transformed hairy roots of *Panax ginseng* by heat treatment.

MATERIALS AND METHODS

Culture of hairy roots Root segments excised from three year-old roots of Korean ginseng (*Panax ginseng* C.A. Meyer) were infected with *Agrobacterium rhizogenes* A4 as the same protocol reported by Yang *et al.* [14]. Among several hairy roots lines, KGHR-8A was used as material. Maintenance of hairy roots was performed by transferring the 1.0 g hairy roots into MS [15] liquid medium containing B5 vitamins [16], 3% sucrose in 100 m*l* Erlenmyer flask by 4 weeks of subculture intervals (23°C under complete dark).

Steam heat treatment of hairy roots Hairy roots were washed

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twice with sterilized distilled water and then kept on the iron mesh tray for removing water. Thereafter hairy roots were transferred to the steam heater. Temperatures and times of heat treatment was performed at 105°C for 30, 60, 120, and 180 min, 121°C for 30 and 120 min. The heat-treated roots were dried in oven at 60°C.

Extraction and analysis of ginsenoside

Extraction of ginsenoside was followed by the method of Ando *et al.* [17]. Milled powder (1 g) of hairy roots were socked in 80% MeOH at 60°C. After evaporation, the residue were dissolved in H_2O and washed twice with ether followed by extraction with n-BuOH saturated with water. The BuOH layer was evaporated to give crude saponins. Each sample was dissolved in EtOH and this liquid were filtrated and subjected to HPLC. For TLC, total ginsenosides (5 μl) was spotted together with the standard samples on TLC plate (silica gel 60 F_{254}, Merck) containing $CHCl_3$-MeOH-H_2O (65:35:10, v/v), thereafter stained by spraying with 30% H_2SO_4, followed by heating at 105°C. Ginsenoside were analyzed using HPLC (Model) on LiChrospher-NH_2 column (Merk Co., 10 μm, 0.46 cm I.D× 25 cm) in CH_3CN-H_2O-BuOH (80:20:10, v/v) and CH_3CN-H_2O (90:10, v/v) with monitoring using refractive index (RI) (Waters R401) at 202 nm. Flow rate was 0.3 ml/min for the former and 2.0 ml/min for the latter. Each ginsenoside was calculated by comparison with the authentic ginsenoside provided from Korean Ginseng and Tobacco Research Institute. Quantitative analysis was performed by the one-point curve method by external standards of authentic ginsenosides and the data were expressed in mg/g dry wt.

RESULTS AND DISCUSSION

When one gram of hairy roots of KGHR-8A line was cultured on MS medium for 4 weeks, 430 mg of dry weight of roots was produced. About 15 mm long hairy root grew to 87 mm after 4 weeks of culture and the number of lateral roots was 19 (Figure 1).

In the line of hairy roots (KGHR-8A), the content of Rg_1 (19.67 mg/g dry wt) was exceptionally high compared to normal hairy roots (3.43 mg/g dry wt), indicating highly useful for the utilization of Rg_1 · for chemical and medicinal purpose. The other ginsenosides showed similar content to the normal hairy roots.

Heat treatment of hairy roots was resulted in the changes in the content and composition of ginsenoside compared to freeze-dried roots (Figure 2, Table 1). Contents of ginsenoside-Rb_1, Rb_2, Rc, Rd, Re, Rf, Rg_1 were decreased in heat treated-roots (Table 1). Ginsenoside-Rg_3, -Rh_1 and -Rh_2 are detected as trace amount in ginseng roots without heat treatment, but heat treatment strongly stimulated the production of those ginsenoside (Table 1). The production of each ginsenoside was depended on the treatment of temperature and its duration (Table 1). Optimum heat treatment to produce the ginsenoside-Rg_3, -Rh_1

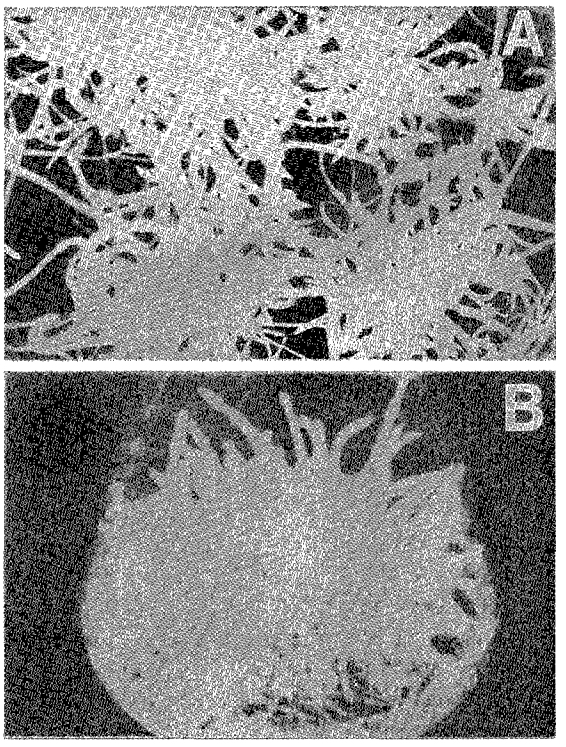

Figure 1. Hairy roots (KGHR-8A) selected from *Agrobacterium*-transformed ginseng hairy roots, KGHR-8. A. Hairy roots cultured in 1/2 MS solid(A) and liquid(B) media with 3% sucrose in Petridish and 100 ml Erlenmyer flask.

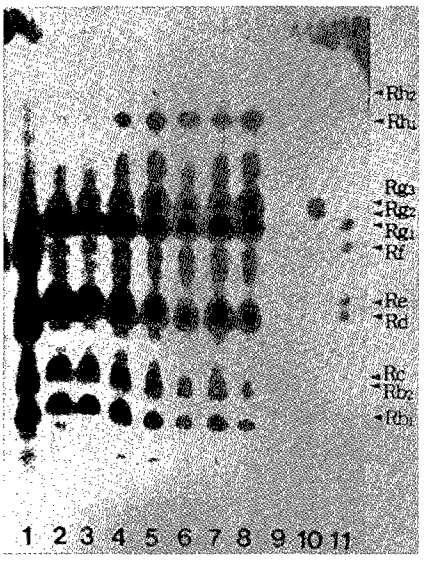

Figure 2. TLC profiles of crude ginseng saponins by BuOH extration from hairy roots (KGHR-8A) with and without heat treatment. Mobile phase: $CHCl_3$:MeOH:H_2O (65:35:10, v/v, lower phase). Lanes 1: freeze dried hairy roots, 2: heating for 60 min at 90°C, 3: heating for 30 min at 105°C, 4: heating for 1 hour at 105°C, 5: heating for 2 hours at 105°C, 6: heating for 3 hours at 105°C, 7: heating for 30 min at 121°C, 8: heating for 60 min at 121°C, 9: ginsenoside-Rh_1, Rh_2 standard, 10: ginsenoside-Rg_2, Rg_3 standard, 11: ginsenoside-Rb_1-Rg_1 standard.

Table 1. Change of ginsenoside composition according to steam heat treatment in ginseng hairy roots.

	Temp (°C)-time (min)	Ginsenoside composition %/total ginsenoside												
		Rb_1	Rb_2	Rc	Rd	Re	Rf	Rg_1	Rg_2	Rg_3	Rh_1	Rh_2	PD/PT	Total mg/g dry wt
Natural fine root	No treatment	22.93	15.28	20.06	7.32	23.23	2.67	3.43	5.09	T	T	0	1.91	69.78
Transformed hairy root	No treatment	20.92	14.64	6.43	7.13	25.25	1.26	19.67	4.70	T	T	0	0.97	27.05
	105-60	17.26	11.71	4.80	6.16	20.83	1.06	18.94	8.44	2.13	6.88	1.79	0.78	22.30
	105-180	13.15	8.50	3.25	4.80	14.85	1.78	12.53	21.50	3.25	13.61	2.78	0.56	12.93
	121-60	10.83	5.92	2.92	3.77	13.53	2.15	12.45	21.98	7.46	14.22	4.77	0.55	13.01

All roots were freeze-dried except for steam heat treatment.
Data were the mean of three independent experiments.
T: Trace amount, PD: Ginsenoside-Rb_1+Rb_2+Rc+Rd+Rh_2+Rg_3, PT: Ginsenoside-Re+Rf+Rg_1+Rg_2+Rh_1.

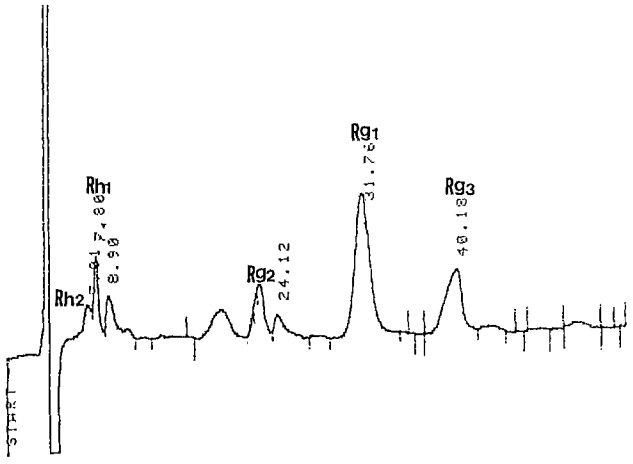

Figure 3. HPLC profile of crude ginseng saponins by BuOH extration from hairy roots with heat treatment for 2 hours at 105°C. HPLC conditions: RI detector, LiChrospher-NH_2 column (4.6x 250 mm), CH_3CN/H_2O/BuOH (80:20:10, v/v).

and -Rh_2 was 105°C for 2 hr and the amount was 2.58, 3.62, and 1.08 mg/dry wt, respectively (Table 1, Figure 3). Amount of ginsenoside-Rg_2 was increased by treatment at 105°C and 121°C. In heat treatment at 105°C for 1 hr, content of ginsenoside-Rg_2 was increased markedly at about 4 times (4.92 mg/g dry wt) compared to control (1.27 mg/g dry wt) (Table 1).

In the Red ginseng roots made from natural roots, each content of the Red ginseng specific ginsenoside-Rg_3, -Rh_1 and -Rh_2 was about 0.14, 0.06, and 0.01 mg/g dry wt, respectively (4). Whereas, the heat-treated transformed hairy roots contained exceptionally high amount of the Red ginseng specific ginsenosides, about more than 10 to 100 times compared to natural Red ginseng. The red ginseng has some superior pharmacological effects compared to fresh or dried ginseng roots [3], showing strong anti-tumor activities because of the Red ginseng specific ginsenosides [5,9-13]. These specific ginsenosides can be used for important medicine. However, practical use of Red ginseng specific ginsenosides is very difficult because of high price. The *Agrobactrium*-transformed hairy roots (KGHR-8A) might be highly applicable for the production of the Red ginseng specific ginsenosides for medicinal purpose.

Ginseng saponin is categorized into triterpenoid as a group of dammarane type and differenciated into panaxadiol and panaxatriol aglycone depended on the number of OH (Park, 1996). Each ginsenoside is determined by combination of Glucose, rhamnose, xylose, or arabinose to OH at the site of C-3, C-20, C-6 or C-20 [3]. In freeze dried roots, the ratio of panaxatriol-ginsenoside (PT) to panaxadiol ginsenoside (PD) was 0.97 but in heat treated roots at 105°C for 2 hr was 0.77. This change might be resulted from increase of the ginsenoside-Rg_2 and Rh_1 as PT. To produce Red ginseng specific ginsenosides (ginsenoside-Rg_3, Rh_1 and Rh_2), the most effective condition of heat treatment was 105°C for 2 hr. The production of ginsenoside-Rg_2, -Rg_3, -Rh_1 and -Rh_2 might be caused from the hydrolysis reaction during heat treatment. Ginsenoside-Rb_1, -Rb_2, -Rc, -Rd were converted into 20 (R and S)-ginsenoside Rg_3, and Rh_2 [2,7]. Ginsenoside-Re was converted to 20 (R and S)-ginsenoside-Rg_2 and Rh1 [8]. It was accepted that the production of ginsenoside-Rg_2, Rg_3, Rh_1, Rh_2 might be resulted from the various chemical transformation such as epimerization and hydroxylation during the process of steam heat treatment.

Conclusively high amount of Red ginseng specific ginsenosides (ginsenoside-Rg_2, Rg_3, Rh_1, Rh_2) were produced from the hairy roots by steam heat treatment.

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