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ORIGINAL PAPER

Dynamics of ginsenoside biosynthesis in suspension culture of *Panax quinquefolium*

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Abstract Biosynthesis of six saponins (ginsenosides) in suspension culture of *P. quinquefolium* Z_5 was investigated. Ginsenoside content in biomass reached the highest level, nearly 30 mg g^{-1} d.w., between 25 and 30 days of the culture. Saponins were synthesized simultaneously with cell growth but their synthesis rate was not proportional to the growth rate. During the phase of rapid biomass multiplication, after which biomass reached 90% of its maximum yield, only half examined ginsenosides was produced. The second half of the final saponins yield was produced during the slow growth phase, in which only 10% of biomass was grown. During the intensive growth phase the productivity of six saponins examined per biomass (dry weight) unit was 3.4 μg mg^{-1} d.w. day^{-1}, however, this parameter calculated for slow growth phase reached nearly 30 μg mg^{-1} d.w. day^{-1}. There were differences in increase of the contents of six saponins determined in biomass, and it was the highest for saponins Re (20(*S*)-protopanaxatriol-6-[*O*-α-L-rhamnopyranosyl(1 → 2)-β-D-glucopyranoside]-20-*O*-β-D-glucopyranoside) and Rg_1 (20(S)-protopanaxatriol-6,20-di-*O*-β-D-glucoside).

Keywords Ginseng · In vitro · Cell culture · Panaxosides

Introduction

A North American *Panax*—*P. quinquefolium* (*Araliaceae*), similarly to the most famous known representative of the *Panax* genus—*P. ginseng*, contains ginsenosides—triterpene saponins. These metabolites are responsible for medical activity of *Panax*, i.e., adaptogenic effect and antistress effect (Rai et al. 2003; Lee et al. 2006), neuroprotective activity (Lian et al. 2005; Naval et al. 2007), immunological activity (Belegortsera et al. 2000; Keith and Block 2003; Predy et al. 2005). Ginsenosides (panaxosides) are glycosides in which aglycon part may be: protopanaxadiol, protopanaksatriol or oleanolic acid. A carbohydrate part is built from 3 to 6 residues of sugars, such as glucose, arabinose, rhamnose, xylose or glucuronic acid. In the present article, dynamics of ginsenosides production in the cell suspension culture of *P. quinquefolium* Z_5 is presented.

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Materials and methods

Plant material

Plant material used for initiation of in vitro cultures of *Panax quinquefolium* originated from the plantation of the University of Agriculture in Lublin (Poland). Seeds of *P. quinquefolium* used for the plantation initiation were supplied by Prof. Bryan F. Zilkey (Dehli Station, Ontario, Canada), where this plant was identified. Suspension culture of the selected cell line Z_5 of *P. quinquefolium* was initiated from callus after IX passage, growing in dark on medium MS with 2,4-D 1 mg/l, NAA 1 mg/l, BAP 0.5 mg/l and 50 g/l sucrose.

Culture conditions

Suspension culture was conducted in Erlenmeyer flasks (300 ml) containing 50 ml of a liquid MS medium with 2,4-D 0.2 mg/l and TDZ 0.002 mg/l, pH 5.6–5.8. The

medium was sterilized in temperature of 123°C and 1 atm pressure for 16 min. The flasks were placed on a rotary shaker (100 rpm), 26 ± 2°C temperature, 90% humidity, light 40 μE m^{-2} s^{-1} for 40 days. Average fresh biomass of inoculum was 9.56 g/l, and average dry weight was 0.86 g/l.

Biomass from 5, 10, 15, 20, 25, 30, 35 and 40 days of cultivation was separated through filtration (using vacuum pomp) and dried at a room temperature. This material was used for ginsenosides extraction.

Extraction procedure

Samples of 1 g of dry raw material (weighed to 0.1 g tolerance) were placed in the 250 ml flasks. They were extracted three times with 50 ml of 80% methanol for 30 min at the solvent's boiling temperature under reflux condenser. Combined methanol extracts were evaporated until dryness in vacuum evaporator under lowered pressure at 60°C. The flask with dried residues was placed in a desiccator filled with a drying agent. The dried methanol extract was weighed.

Ginsenoside analysis using HPLC methods

Dried extracts were dissolved in 2 ml of methanol (HPLC grade) and filtered through a 0.2 μm pore diameter Millex®-FG Hydrophobic Fluoropore filters (PTFE). Then aliquots of 20 μl were introduced to liquid chromatography system consisting of LiChroART® 250-4, Waters 600 Controller pump and UV–Vis Waters 996 detector combined with Pentium 60 PC hardware equipped with Millenium software. Two different mixtures of acetonitrile with water were used as eluent. Acetonitrile to water ratio 30:70 was used for determination of the ginsenosides Rb_1, Rb_2, Rc, Rd (flow rate 2 ml/min, analysis time 45 min), and 18:82 ratio was used for determination of the ginsenosides Rg_1 and Re (flow rate 3 ml/min, analysis time 40 min). Ginsenoside detection was made at 203 nm wavelength.

Results

The presence of six ginsenosides, i.e., Rb_1, Rb_2, Rc, Rd, Re and Rg_1 in biomass of growing cell culture of *P. quinquefolium* Z_5 was determined. Content of ginsenosides in 1 g of dry biomass increased reaching the highest level after 25–30 days culture. The content of two metabolites Rg_1 and Re (both belong to the Rg group) showed the highest increase from about 2.8 to 7.6 mg g^{-1} d.w. and 3.4 to 8 mg g^{-1} d.w., respectively. Contents of the other saponins had minor increase dynamics—from 0.8–1.6 mg g^{-1} d.m. to 2.4–4.8 mg g^{-1} d.w. (Table 1). The total content of examined ginsenosides increased nearly 3-fold from over 11 mg g^{-1} d.m. to over 29 mg g^{-1} d.m. Prolongation of the *P. quinquefolium* culture beyond the growth phase caused great decrease of the saponin level (Figs. 1, 2).

Dynamics for both the cell growth and saponins biosynthesis was determined (Fig. 1). Ginsenosides production accompanied the biomass increase, however, during the most intensive biomass growth between 10 and 20 days of culture (linear growth phase G_1, $\Delta X = 5.2$ mg/ml, over 76% final biomass yield) only about 43% of the final saponin yield was produced ($\Delta P = 85.7$ μg/ml) (Table 2). After that, during the slow growth phase, the biomass increase was 0.65 mg/ml only, but ginsenoside production was very efficient ($\Delta P = 97.4$ μg/ml) what constituted nearly 50% of its final yield. After the biomass growth was stopped the saponin contents dropped (Table 2).

Table 1 Content of ginsenosides in suspension culture (MS, 2.4-D 0.2 mg/l; TDZ 0.002 mg/l)

Culture time	Ginsenosides (mg/g d.m.) ± SE								
	Rb_1	Rb_2	Rc	Rd	Re	Rg_1	Rb group	Rg group	Total
5	0.77 ± 0.13	1.18 ± 0.33	1.59 ± 0.25	1.36 ± 0.17	3.40 ± 0.11	2.84 ± 0.11	4.9	6.24	11.14
10	1.59 ± 0.19	1.93 ± 0.20	1.65 ± 0.11	1.50 ± 0.65	4.52 ± 0.88	3.85 ± 0.43	5.07	8.37	13.44
15	1.47 ± 0.22	2.03 ± 0.11	1.67 ± 0.31	2.11 ± 0.87	5.07 ± 0.54	3.39 ± 0.51	7.28	8.46	15.74
20	1.50 ± 0.71	2.08 ± 0.31	1.99 ± 0.22	2.26 ± 0.31	4.69 ± 0.33	4.55 ± 0.65	7.83	9.24	17.07
25	2.41 ± 0.32	4.79 ± 0.13	3.03 ± 0.18	4.00 ± 0.33	6.88 ± 0.27	8.07 ± 0.34	14.23	14.95	29.11
30	2.24 ± 0.33	3.94 ± 0.62	3.35 ± 0.59	3.91 ± 0.56	7.59 ± 0.11	6.54 ± 0.12	13.44	14.13	27.57
35	1.40 ± 0.27	2.03 ± 0.28	1.94 ± 0.33	2.17 ± 0.43	6.24 ± 0.31	4.26 ± 0.43	7.54	10.5	18.04
40	1.45 ± 0.11	2.21 ± 0.14	1.88 ± 0.72	2.56 ± 0.23	4.76 ± 0.71	4.23 ± 0.52	8.1	8.99	17.09

In table, averages from 6 repetitions are placed. Group Rb—sum of protopanaxadiol derivatives: Rb1, Rb2, Rc, Rd. Group Rg—sum of protopanaxatriol derivatives: Rg1 and Re

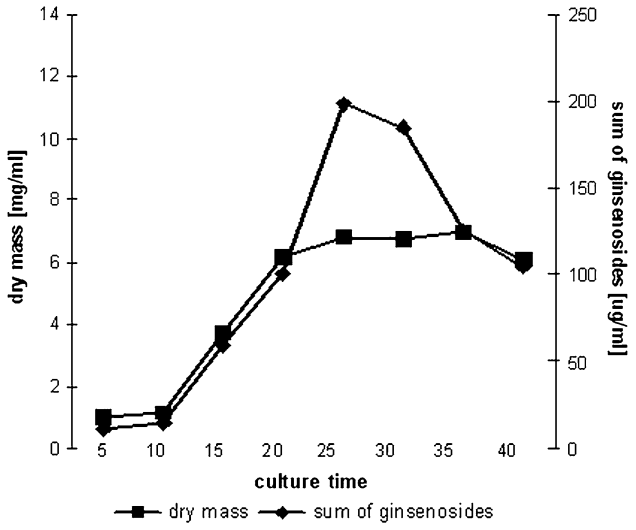

Fig. 1 Dynamics of biomass growth and saponin biosynthesis during 40-days of suspension culture of *P. quinquefolium* Z_5

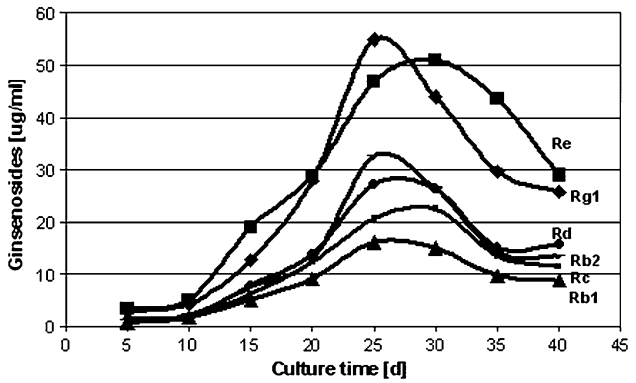

Fig. 2 Dynamics of individual ginsenosides biosynthesis in suspension culture of *P. quinquefolium* Z_5

Discussion

Ginsenosides content in biomass reached the greatest level in 25 days of cultivation. The level of all examined metabolites in biomass increased almost 3-fold, and was the highest for saponins Re and Rg_1 (Table 1; Fig. 2). Saponin biosynthesis and growth of biomass during the process proceeded simultaneously but their dynamics was different. For the rapid growth phase (G_1) between 10 and 20 days, 90% of total biomass yield and only 50% of product was accumulated with low synthesis rate of about 3.4 μg mg^{-1} d.w. day^{-1}. The saponin content reached about 17 μg mg^{-1} d.w. in this phase. The rest of the product (50%) was accumulated with high rate nearly 30 μg mg^{-1} d.w. day^{-1} in the slow growth phase (G_2) in which biomass increased by only 10%. The saponin content reached maximum nearly 150 μg mg^{-1} d.w. in this phase (Table 2). These relationships clearly show, that although saponins are synthesized already simultaneously with the cell growth, their synthesis rate is not proportional to the growth rate (Fig. 3).

Some decrease of ginsenoside contents was observed after biomass growth has been stopped. It can be assumed that it is a result of the activities of enzymes involved in saponin metabolism. Moreover, it is probably that lower saponins content in biomass is due to a cell lysis facilitating ginsenoside extraction into medium. Mathur et al. (1994) examined that amount of various fractions of ginsenosides decrease in biomass, but increased in the medium after 25 days cultivation of *P. quinquefolium* suspension.

For a culture of *P. ginseng* dynamics of saponin production was dependent on mineral nutrients. Liu and Zhong (1996, 1997) had presented the similar course of the process as in our study, and they also showed saponins production (20–50 mg g^{-1} d.w.) after cell multiplication; in stationary phase was stopped, what was depended on both N-source and potassium concentration. Akalezi et al. (1999) have shown that saponin content initially decreases and afterwards keeps constant level independent on inoculum size with 30 g/l saccharose in medium. When sugar content was increased to 60 and 80 g/l and inoculum to 3 g/l intensive ginsenosides biosynthesis (maximum 0.275 g/l) was observed at the beginning (10–15 days of cultivation time) of a phase of biomass growth (5–20 days of cultivation time). Khodakovsaya et al. (1998) showed that maximum saponin content in different *P. ginseng* lines

Table 2 Data composition from the process of saponin biosynthesis in suspension culture *P. quinquefolium* Z_5

Culture days	Growth phase	Dry mass (mg/ml)	ΔX (mg/ml)	Ginsenosides (μg/ml)	ΔP (μg/ml)	$\Delta P/\Delta X$
5	L	1.01		11.3		
10		1.13	0.12	14.8	3.5	29.16
15	G_1	3.75	2.62	59.1	44.3	16.91
20		6.15	2.40	100.5	41.4	17.25
25	G_2	6.803	0.653	197.9	97.4	149.15
30	S	6.72		185.2		
35		6.97		125.7		
40	D	6.085		104.1		

L lag phase, G_1 intensive growth phase, G_2 slow growth phase, *S* stationary phase, *X* biomass, ΔX biomass increase, *P* product (ginsenosides), ΔP product increase (ginsenosides), *D* growth decline phase

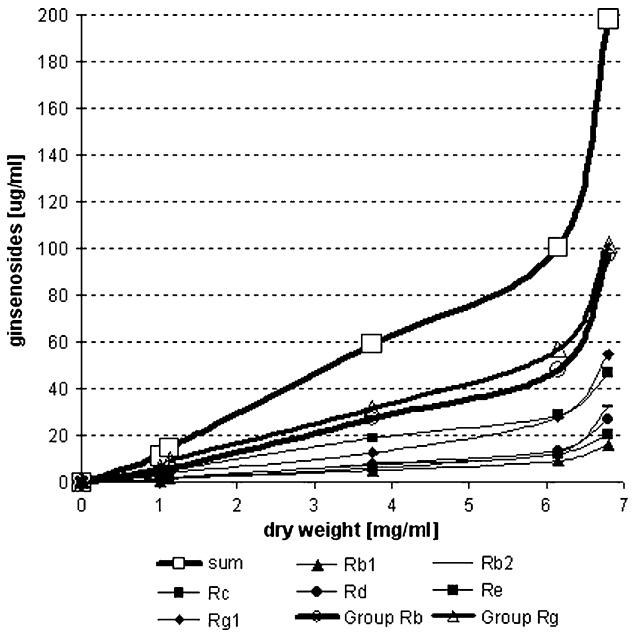

Fig. 3 Graphical interpretation of the relationship between ginsenoside production per 1 ml of culture and biomass growth per 1 ml of culture in the suspension culture of *P. quinquefolium* Z_5. Group Rb—sum of protopanaxadiol derivatives: Rb1, Rb2, Rc, Rd; Group Rg—sum of protopanaxatriol derivatives: Rg1 and Re

Table 3 Ginsenoside contents in suspension cultures of different species of *Panax*

Species of *Panax*	Ginsenoside content	References
P. quinquefolium	1.50 g/l	Zhong and Wang (1998)
P. ginseng	0.73 g/l	Liu and Zhong (1996)
P. ginseng	0.60 g/l	Liu and Zhong (1997)
P. ginseng	17 mg/g d.w.	Khodakovsaya et al. (1998)
P. ginseng	0.28 g/l	Akalezi et al. (1999)
P. notoginseng	1.19 g/l	Zhong and Wang (1996)
P. notoginseng	1.77 g/l	Zhang et al. (1996)
P. notoginseng	0.92 g/l	Zhong et al. (1999)

growing in dark or light or with addition of ginsenoside precursors into medium or transformed lines, was oscillating between 7 and 17 mg g^{-1} d.w. According to Zhong and Wang (1998) during the growth phase (between 10 and 35 days of cultivation) of *P. quinquefolium* suspension culture ginsenoside synthesis had a slow rate, which clearly increased to 1.5 g/l (measured by colorimetric method), after 35 days of the culture.

For *P. notoginseng* the dynamics of saponin synthesis was dependent on culture condition. It may occur at the beginning of culture when there is a high sugar concentration in medium (60 g/l) content of total saponins (measured by colorimetric methods) was above 160 mg in 1 g of biomass (Zhang et al. 1996) or they are synthesized (above 900 mg/l for total saponin according to TLC colorimetry) simultaneously to a biomass growth at saccharose concentration of 30 g/l (Zhong et al. 1999) (Table 3).

It is interesting that the maximum content of saponins in the suspension culture investigated in this study (29 mg g^{-1} d.w.) was comparable to their contents in the leaves of 3-years-old plants of *P. quinquefolium* (24 mg g^{-1} d.w.) from a field cultivation (Kochan et al. 2008) which were the source of explants for our in vitro cultures initiation. The quantitative pattern of protopanaxadiol derivatives was the same: $Rb_2 > Rd > Rc.Rb_1$, however, opposite results were found for protopanaxatriol derivatives: $Rg_1 > Re$ for the suspension culture and $Re > Rg_1$ for the leaves.

Comparing saponin contents in our suspension culture and in the roots from field cultivation, which are pharmaceutical raw materials. The roots of 3-years-old plant contained much more ginsenosides—about 56 mg g^{-1} d.w. (Kochan et al. 2008). Saponins pattern was entirely different in roots ($Rb_1 > Rc > Rd > Rb_2$ and $Re > Rg_1$) than in our suspension culture ($Rb_2 > Rd > Rc > Rb_1$ and $Rg_1 > Re$).

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