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Effects of Polyacetylenes from *Panax ginseng* on Some Microsomal and Mitochondrial Enzymes

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Abstract—Effects of panaxydol, panaxynol and panaxytriol isolated from *Panax ginseng* C.A. Meyer on some enzyme activities were determined. Activities of ATPase, membrane-bound enzyme from Sarcoma 180 and rat liver were slightly inhibited by panaxydol. Activities of 5′-nucleotidase, membrane-bound enzyme and succinate cytochrome c reductase in mitochonidria from Sarcoma 180 and rat livers were significantly inhitited in a dose-dependent manner by panaxynol. The inhibitory effects of panaxydol and panaxynol on succinate cytochrome c reductase activities were more potent than those on 5′-nucleotidase activities and panaxynol was found to be a very potent inhibitor of succinate cytochrome c reductase. Activities of glucose-6-phosphatase in endoplasmic reticulum from Sarcoma 180 and rat livers were not affected by all three polyacetylenes. These results suggested that the inhibitory effects of panaxydol and panaxynol on enzyme activities might contribute to their biological activities.

Keywords—*Panax ginseng* • polyacetylenes • biological activity • panaxydol • panaxynol • panaxytriol • ATPase • 5′-nucleotidase • glucose-6-phosphatase • succinate-cytochrome c reductase

Among polyacetylenes isolated from *Panax ginseng* C.A. Meyer, panaxynol was firstly isolated by Takahashi[1,2]. Panaxydol and panaxytriol were reported by Poplawski[3] and Shim[4]. Ahn[5] found that panaxydol had a very strong cytotoxic activity against L1210 cell. This finding was followed by isolations of series of polyacetylenes including C_{17}- and C_{14}-polyacetylenes which showed cytotoxicities against L1210 cell[6~11]. Also, panaxytriol inhibited the growth of murine lukemia and human cancer cells[12], panaxacol and dihydropanaxacol isolated from Ginseng callus showed cytotoxicity against Yoshida Sarcoma 180 cell[13]. It was reported that petroleum ether extract of ginseng inhibited the growth of some cancer cell lines[14~16] and inhibited macromolecular synthesis of Sarcoma 180, L5178Y, L1210 and human cancer cells [16,17]. Recently the active substances responsible for these effects have been known as polyacetylenes.

In the previous studies[18,19], panaxydol, panaxynol and panaxytriol inhibited DNA, RNA and markedly protein synthesis and it was suggested that polyacetylenes might regulate the growth of cell by elevation of intracellular cyclic AMP and their cytotoxicities to cancer cells might be releated to membrane damage.

As for biological and pharmacological activities of polyacetylenes, it was reported that panaxynol isolated from Ginseng roots showed anti-inflammatory effect[20] and falcarindiol, heptadeca-1, 8-dien-4, 6-diyn-3, 10-diol and panaxynol isolated from Saposhnikovae radix inhibited the formation of HHT and thromboxan B_2 in human platelets[21] and falcarindiol isolated from *Schettlera diginata* exhibited anti-fungal activity with destruction of the plasma membrane of dermatophytes[22]. Inhibition of lipid peroxidation and protection of liver from CCl_4 intoxication by panaxydol, panaxynol and panaxytriol were also reported recently[23,24]. These polyacetylenes supressed the osmotic behavior of liposomes composed of phosphatidyl choline and phosphatidic acid and panaxydol and panaxynol caused erythrocyte hemolysis dose-dependently[25].

Since we expected that cytotoxic actions of polyacetylenes might be due to their effects on some microsomal or mitochondral enzyme activities, the effects of panaxydol, panaxynol and panaxytriol on activities of ATPase and 5′-nucleotidase in plasma membrane, glucose-6-phosphatase in endoplasmic reticulum and succinate cytochrome c reductase in mitochondria from murine Sarcoma 180 and rat livers were studied in the present report.

Experimental

Animals

Male ICR mice(20~22 g) and male Sprague-Dawley rats(160~180 g) were supplied from Experimental Animal Laboratory of Korea Ginseng & Tobacco Research Institute.

Chemicals

Sodium citrate, cytochrome c, sucrose, EDTA, sodium deoxycholate, ATP, glucose-6-phosphate, succinate disodium salt and 5′-nucleotidase kits were purchased from Sigma Chemical Co. Tris(hydroxy methyl) aminomethan, ammonium molybdate and TCA were purchased from Junsei Chemical Co.

Isolation of polyacetylenes

Panaxydol, panaxynol and panaxytriol were isolated from petroleum ether extract of Red Ginseng and identified according to the method described previously[19]. The chemical structures of polyacetylenes are shown in Table I.

Preparations of enzyme sources

1×10^6 Sarcoma cells were transfered to the peritoneal cavities of ICR mice. After 8~10 days, Sarcoma 180 cells were collected from ICR mouse, washed five times with ice-cold physiological saline and were homogenized at 1,000 rpm for 10 min in 5 volume of 38 mM Tris-HCl buffer(pH 7.4) containing 15 mM EDTA, 0.25 M sucrose and 2.4 mM sodium

Table I. Structures of polyacetylene compounds

Compound	Structure
Panaxydol	$CH_2=CH-\underset{\underset{OH}{\vert}}{CH}-(C\equiv C)_2-CH_2-\underset{\diagdown\ \diagup}{CH-CH}-(CH_2)_6-CH_3$ (O)
Panaxynol	$CH_2=CH-\underset{\underset{OH}{\vert}}{CH}-(C\equiv C)_2-CH_2-CH=CH-(CH_2)_6-CH_3$
Panaxytriol	$CH_2=CH-\underset{\underset{OH}{\vert}}{CH}-(C\equiv C)_2-CH_2-\underset{\underset{OH}{\vert}}{CH}-\underset{\underset{OH}{\vert}}{CH}-(CH_2)_6-CH_3$

deoxycholate for both ATPase and 5′-nucloptidase assay, and 10 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA and 0.25 M sucrose for both glucose-6-phosphatase and succinate cytochrome c reductase assay. The cellular homogenate was centrifuged at 300 g for 30 min. The resulting supernatant was centrifuged at 4,000 g for 30 min. The 4,000 g pellet as a mitochondrial fraction was washed twice with homogenizing buffer, and resuspended in 50 mM sodium phosphate buffer (pH 7.4) for the assay of succinate cytochrome c reductase. The 4,000 g supernatant was further centrifuged at 20,000 g for 30 min. The obtained pellet as a microsomal fraction was resuspended in 38 mM Tris-HCl buffer (pH 7.5) containing 15 mM EDTA and 0.25 M sucrose for the assay of ATPase and 6′-nucleotidase or 10 mM Tris-HCl buffer(pH 7.4) containing 0.25 M sucrose for the assay of glucose-6-phosphatase.

Rat livers were homogenized in 5 volume of each homogenizing buffer and centrifuged at 900 g for 10 min. The supernatant was centrifuged at 13,000 g for 20 min. The pellet was used as a mitochondrial fraction and the supernatant was further centrifuged at 100,000 g for 1 hour. The 100,000 g pellet was used as a microsomal fraction. The homogenizing and resuspending buffers using for the assay of each enzyme activities were same as described above.

Proteins were measured by the method of Lowry et al.[26] using bovine serum albumin as a standard.

Determination of enzymatic activities

ATPase activity was determined by the method of Phillips et al.[27]. A 1 ml of assay mixture contained; 100 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, 1 mM ATP, 100 mM Tris-HCl buffer(pH 7.5) and 100 μg of enzyme protein. The reaction mixture was preincubated at 37° for 5 min. The reaction was started by the addition of microsomal fraction to assay mixture and stopped after 10 min with 1 ml of ice-cold 15% TCA. The precipitate formed was removed by centrifugation after the tubes stood in an ice-bath. A 0.5 ml aliquot was pipetted off and the liberated inorganic phosphate in supernatant was determined according to the method of Horwitt[28].

5′-Nucleotidase activity was determined by using a commercial kit based on an enzymatic kinetic method reported by Arkesteijn[29].

Glucose-6-phosphatase activity was assayed according to the method of Swanson[30]. A 0.5 ml of reaction mixture contained; 0.1 ml of 0.1 M glucose-6-phosphate, 0.3 ml of 0.1 M citrate buffer(pH 6.5) and 0.1 ml of the diluted enzyme solution (100 μg of enzyme protein). Assay mixtures were preincubated at 37° for 5 min. The reaction was initiated by the addition of microsomal fraction to the reaction mixtures. After 10 min the incubation was terminated by the addition of 1 ml of ice-cold 10% TCA. The liberated inorganic phosphate was measured by the method of Horwitt[28].

Succinate cytochrome c reductase activity was determined by the method of Mackler et al.[31]. The complete system contained; 20 μl of 100 mM KCN, 50 μl of 1.25% cytochrome c, 50 μl of mitochondria fraction (50 μg of enzyme protein) and 1 ml of 50 mM phosphate buffer (pH 7.4). All reagents were made up in the phosphate buffer. The reaction was started by the addition of enzyme solution. The molar extinction coefficient of reduced cytochrome c at 550 nm was calculated as 27,000.

Polyacetylenes dissolved in 100% ethanol and final ethanol concentration was below 0.2% in the reaction mixtures. The effects of polyacetylenes on the enzyme activities were determined by including the compounds being tested at the desired concentration in the reaction mixture.

Results and Discussion

Effects of polyacetylenes on ATPase activity

Activities of ATPase, membrane-bound enzyme from Sarcoma 180 and rat livers were inhibited by 10~20% at a concentration of 20~30 μg/ml of panaxydol(Table II, III). But panaxynol and panaxytriol had no effect on ATPase activities from Sarcoma 180 and rat livers.

Racker and Coworkers[32~34] proposed that increase in ATPase activity in different pump system, may be the reason for the elevation of aerobic glycolysis in various malignant cells. Quercetin is also known as a potent inhibitor of protein synthesis and cell growth and inhibited the high aerobic glycolysis in tumor cells. It was also suggested that inhibition of high aerobic glycolysis in tumor cells. It was also suggested that inhibition of high aerobic glycolysis is due to the effect of quercetin on different ATPase systems.

Panaxydol, panaxynol and panaxytriol had no effect on the glycolysis of L1210 cells in the previous studies[19]. On the basis of the above observations. It could be expected that polyacetylenes did not inhibited the glycolysis of L1210 cells since ATPase was slightly inhibited or not affected by polyacetylenes.

Effects of polyacetylenes on 5′-nucleotidase activity

At each concentration of 10, 20 and 30 μg/ml, panaxydol and panaxynol significantly inhibited the activities of 5′-nucleotidase, membrane-bound enzyme from Sarcoma 180 and rat livers in a dose-dependent pattern(Table IV, V). The inhibitory effects of panaxydol were more potent that those of panaxynol. However panaxytriol did not show the inhibitory effects.

Table II. Effects of polyacetylenes on ATPase activity in microsome from Sarcoma 180 cells

Concentration (μg/ml)	Polyacetylenes		
	Panaxydol	Panaxynol	Panaxytriol
None	1.04±0.04(100)	1.04±0.04(100)	1.04±0.04(100)
10	1.04±0.03(100)	1.04±0.08(100)	1.04±0.07(100)
20	0.94±0.08 (90)*	1.00±0.04 (96)	1.06±0.10(102)
30	0.83±0.08 (80)**	0.95±0.08 (91)	0.98±0.08 (94)

Each value represents mean±S.E. specific activity(μmole pi/mg of protein/10min) of 6~8 determinations at various concentrations. Values in parentheses represent % of control activity.
* $p<0.05$ ** $p<0.005$

Table III. Effects of polyacetylenes on ATPase activity in microsome from rat livers

Concentration (μg/ml)	Polyacetylenes		
	Panaxydol	Panaxynol	Panaxytriol
None	0.88±0.05(100)	0.88±0.05(100)	0.88±0.05(100)
10	0.84±0.02 (95)	0.86±0.02 (98)	0.86±0.07 (98)
20	0.70±0.08 (80)**	0.84±0.06 (95)	0.84±0.09 (95)
30	0.68±0.07 (77)**	0.84+0.04 (95)	0.83±0.04 (94)

Each value represents mean±S.E. specific activity(μmole pi/mg of protein/10min) of 6~8 determinations at various concentrations. Values in parentheses are represent % of control activity.
** $p<0.005$

Table IV. Effects of polyacetylenes on 5′-nucleotidase activity in microsomes from Sarcoma 180 cells

Concentration (μg/ml)	Polyacetylenes		
	Panaxydol	Panaxynol	Panaxytriol
None	23.48±3.48(100)	23.48±3.48(100)	23.48±3.48(100)
10	13.65±2.26 (58)**	13.99±1.74 (60)**	25.22±2.62(107)
20	10.08±1.74 (43)**	13.05±0.87 (56)**	24.34±0.87(104)
30	8.86±1.74 (38)**	10.68±5.22 (45)**	25.22±4.35(107)

Each value respresents mean±S.E.(U/g of protein) of 6~8 determinations at various concentrations. Values in parentheses represent % of control activity.
** $p<0.005$

Table V. Effects of polyacetylenes on 5′-nucleotidase activity in microsomes from rat livers

Concentration (μg/ml)	Polyacetylenes		
	Panaxydol	Panaxynol	Panaxytriol
None	32.07±4.11(100)	32.07±4.11(100)	32.07±4.11(100)
10	23.96±1.95 (75)**	23.20±3.08 (72)**	28.90±1.48 (90)
20	18.64±2.70 (58)**	20.89±1.68 (65)**	30.48±2.52 (95)
30	9.79±1.21 (30)**	14.17±2.81 (44)**	29.79±1.43 (93)

Each value represents mean±S.E.(U/g of protein) of 6~8 determinations at various concentrations. Values in parentheses represent % of control activity.
** $p<0.005$

Table VI. Effects of polyacetylenes on glucose-6-phosphatase activity in microsomes from Sarcoma 180 cells

Concentration (μg/ml)	Polyacetylenes		
	Panaxydol	Panaxynol	Panaxytriol
None	0.18±0.04(100)	0.18±0.04(100)	0.18±0.04(100)
10	0.17±0.01 (94)	0.17±0.01 (96)	0.18±0.02(100)
20	0.18±0.01(100)	0.18±0.01(100)	0.19±0.05(106)
30	0.18±0.02(100)	0.16±0.01 (89)	0.19±0.05(106)
40	0.16±0.04 (89)	0.18±0.04(100)	0.18±0.01(100)

Each value represents mean±S.E. specific activity(μmole pi/mg of protein/10min) of 6~8 determinations at various concentrations.

Inhibitors of membrane-bound enzymes are known to show anti-tumor and immunopotentiator activities[35]. As inhibitors of 5′-nucleotidase, polysaccharides isolated from microbial metabolites showed an anti-tumor activity[36] and phenolic substances isolated from *Areca catecu* exhibited a moderate cytotoxicity to Ehrlich ascites carcinoma strain E, HeLa and HL60 cells, and displayed significant therapeutic activity against Ehrlich ascite carcinoma[37,38].

Therefore, the above results suggest that cytotoxicities of panaxydol and panaxynol to cancer cells might be releated to their inhibitory effects on 5′-nucleotidase activity.

Effects of polyacetylenes on glucose-6-phosphatase activity

Table VII. Effects of polyacetylenes on glucose-6-phosphatase activity in microsomes from rat livers

Concentration (μg/ml)	Polyacetylenes		
	Panaxydol	Panaxynol	Panaxytriol
None	1.57±0.10(100)	1.57±0.10(100)	1.57±0.10(100)
10	1.61±0.04(103)	1.48±0.06 (94)	1.46±0.05 (93)
20	1.59±0.05(101)	1.48±0.06 (94)	1.53±0.05 (97)
30	1.59±0.05(101)	1.48±0.06 (94)	1.50±0.04 (96)
40	1.61±0.05(103)	1.50±0.03 (96)	1.46±0.01 (93)

Each value represents mean±S.E. specific activity(μmole pi/mg of protein/10min) of 6~8 determinations at various concentrations.

Table VIII. Effects of polyacetylenes on succinate-cytochrome c reductase activity in mitochondria from Sarcoma 180 cells

Concentration (μg/ml)	Polyacetylenes		
	Panaxydol	Panaxynol	Panaxytriol
None	76.40±3.15(100)	76.40±3.15(100)	76.40±3.15(100)
5	45.05±1.92 (59)**	21.36±2.74 (28)**	76.40±5.05(100)
10	43.41±1.23 (57)**	20.42±2.60 (27)**	77.77±1.10(102)
15	31.90±3.15 (42)**	13.01±1.51 (17)**	74.62±6.44 (98)
20	18.21±2.60 (24)**	13.14±1.51 (17)**	73.25±6.44 (96)

Each value represents mean±S.E.(μmole succinate oxidized/min/mg of protein) of 6~8 determinations at various concentrations. Values in parentheses represent % of control activity.
** $p<0.005$

Panaxydol, panaxynol and panaxytriol had no effect on the activities of glucose-6-phosphatase in endoplasmic reticulum from Sarcoma 180 and rat livers, even at a concentration of 40 μg/ml of each polyacetylene(Table VI, VII). The basal activity in Sarcoma 180 was 0.18 μmole pi/mg of protein/10 min and extremely low as compared with 1.57 μmole pi/mg of protein/10 min in rat livers.

Effects of polyacetylenes on succinate cytochrome c reductase activity

At each concentration of 5, 10, 15 and 20 μg/ml, panaxydol markedly inhibited the activities of succinate cytochrome c reductase from Sarcoma 180 to 41, 43, 58 and 76% and from rat livers to 27, 59, 70 and 73%, respectively. In case of panaxynol, these enzyme activities from both Sarcoma 180 and rat livers inhibited to about 70% at a concentration of 5 μg/ml(Table VIII, IX). Panaxydol and panaxynol showed the most potent inhibitory effects on succinate cytochrome c reductase among the four enzyme systems tested.

In addition, this enzyme was not affected by panaxytriol which was reported to show least cytotoxicity to L1210 cells and least lysis effect on erythrocytes among the three polyacetylenes[7,25].

Succinate cytochrome c reductase plays a role in inner membrane of mitochondria between the electron transport complex II and complex III. In view of the above results, it was postulated that cytotoxic actions and biological properties of panaxydol and panaxynol might be associated with the inhibition of oxydative phosphorylation.

Table IX. Effects of polyacetylenes on succinate cytochrome c reductase activity in mitochondria from rat livers

Concentration (μg/ml)	Polyacetylenes		
	Panaxydol	Panaxynol	Panaxytriol
None	62.32±3.14(100)	62.32±3.14(100)	62.32±3.14(100)
5	45.26±2.51 (73)**	18.36±1.34 (29)**	60.02±4.08 (96)
10	25.38±1.26 (41)**	13.28±0.86 (21)**	58.54±5.03 (94)
15	18.94±2.04 (30)**	10.61±1.34 (17)**	59.20±3.24 (95)
20	16.97±1.89 (27)**	9.42±1.49 (10)**	61.14±1.57 (98)

Each value represents mean±S.E.(μmole succinate oxidized/min/mg of protein) of 6~8 determinations at various determinations. Values in parentheses represent % of control activity.
** $p<0.005$

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