Biotransformation of Panax notoginseng by fungi

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Abstract**—** *Roots of Panax notoginseng were fermented with 30 fungi respectively. Almost one-third of the products showed increasing antibacterial activity. All products could inhibit GST-CDC25 phosphatase as a potential antitumor agent. HPLC profiles proved that components of unfermented P. notoginseng and fermented P. notoginseng have obviously changes.*

Keywords**— Panax notoginseng, biotransformation, antibacterial activity, antitumor activity.**

I. INTRODUCTION

Panax notoginseng (Sanqi) is a famous traditional Chinese herb. It has obvious functions such as promoting blood circulation, preventing the formation of blood clots, dissolving blood clots, and removing cellular breakdown products or other debris from the blood circulation (Jiang 1995, Li 1999). Microbial fermentation has been used from ancient times in TCM preparation. Microorganisms possess abundant enzyme systems and have strong ability for transforming. The TCM fermented by the microbe may produce new active metabolites, accumulate amount of active components, or modify the structure of existed compounds to be useful. There were few reports to be found concerning the fermentation of *P. notoginseng*. Li *et al.* (2000) has tried to transform ginsenoside Rb1 and Rg1 by pathogenic microbes *Pseudomones* spp. and *Fusarium* spp. to find what occurred after pathogeny. No obvious transformation was observed at the end. Li *et a l.* (2003) reported that Sanqi was fermented by *Bacillus subtilis*. An antitumor compound ginsenodide Rh4 was isolated from the fermentation products, and two saponins, ginsenosides Rh1 and Re had been linked with one more glucosyl moiety. The transformed product of ginsenoside Rh1 was determined to be a new compound as 3-O-β-D-glucopyranosyl-6-O-β-D-glucopyranosyl-20(S)-protopanaxatriol, which was linked with twoβ-D-glucopyranosyls at C-3 and C-6 in the meantime.

In our study, we found that *P. notoginseng* fermented by different fungi showed antibacterial and antitumor activities. It provided us the chance to explore what happened in that processes and the possibility to find new active compounds.

II. MATERIAL AND METHOD

2.1 Fermentation and Extraction

Roots of *P. notoginseng* were purchased from a medicinal herbs market in Yunnan province. Fungi cultures (included *Aspergillus, Mucor, Cyathus, Monascus, Paraniesslia, Ophiobolus, Paecilomgces,* and *Ganoderma*) used to ferment are conserved in the Key Laboratory for Conservation and Utilization of Bio-resource, Yunnan University, Kunming, Yunnan, P.R. China.

P. notoginseng powder (50g) was mixed with 100ml water in a 250ml flask and sterilized for 40 min at 121 ^{o}C. Every flask was inoculated with 10ml culture of a selected strain, which was cultivated for 3 days in a potato dextrose medium. After incubation at 25^{o}C for 15 days, the fermented products were dried in an oven at 60 ^{o}C. The products were extracted three times by methanol, and methanol was removed under vacuum. The residues were dissolved to 10mg/ml with 2% DMSO solution.

2.2 Antibacterial activity assay of extracts

Antibacterial activities against four human pathogenic bacteria (*Escherichia coli*, *Pseudomonas aeruginosa* (ATCC27853), *Staphylococcus aureus* (ATCC25923) and *Shigella. flexneri*) were tested by agar diffusion assay. Suspensions of tested organisms were obtained by incubation in meat broth (0.3% meat extract, 1% peptone, 0.5% NaCl, pH 7.4) with shaking for 2 days at 37 ^{o}C. Extract solution (50µl) were pipeting onto sterilized filter disks (Ø5 mm), which were placed onto the meat extract agar plates homogenized with suspensions of tested organisms. After 18 h, the diameters of the inhibited zones were measured.

2.3 Cytotoxicity assay of extracts

Cytotoxicities of different extracts to the K562 cell (human leukemia) and the A549 cell (human lung carcinoma) were measured using a modified MTT reduction assay (Mosmann 1983). Cells were seeded in a 96-well in a 100ul RPMI-1640 medium. After 24 hours of incubation, tested extracts were added. The final concentration of tested extracts is 100ug/ml and

active control DDP is from 0.1-10ug/ml. After 48 hr (K562) or 72 hr (A549) of culture at 37℃in 5%CO_2 , 20ul of MTT (5mg/ml) solution was added into each well and kept incubating for 4 hr. Then 100ul of 10%SDS-5%isobutanol-0.012mol/l HCL (W/V/V) was added to each well and the plate was incubated overnight at room temperature. Absorbance at 570 and 630 nm was measured with an ELISA plate reader (Bioteck, EL-340, USA).

Each test was repeated at least three times and each microplate had its own control. Each value of different tested extracts and controls was based on the mean of at least 6 wells.

2.4 Assay of GST-CDC25 phosphatase activity

Assays were performed in microtitration plates as described by Baratte (Loukaci 2001). GST-CDC25 (20mL) was added to 20mL of 100mM DTT in Tris buffer (50mM Tris, pH8.0, 50mM NaCl, 1mM EDTA, 1mM DTT) and 140 mL of Tris buffer A. Plates were pre-incubated at 37℃ for 15 min in a DenleyWell warm microplate incubator. Reactions were initiated by addition of 20 mL of 500 mM p-nitrophenylphosphate in Tris buffer. After 30 min incubation at 37 ℃, absorbance at 405 nm was measured in a Biorad microplate reader.

2.5 HPLC analysis of extracts

HPLC was performed with an Agilent 1100 system (Agilent, USA). Analysis was achieved by using an SB-C18 column (partical size 5um, 4.6mm×250mm, Agilent, USA). Samples with a linear gradient were eluted with methanol from 5% to 100% at a flow rate of 1.0ml/min in 60min. Each ultraviolet spectrum (in the range 190-600 nm) was recorded with DAD detector.

III. RESULTS

3.1 Antibacterial activity of P. notoginseng extracts fermented by selected fungi

P. notoginseng fermented by different fungi showed certain activity under test conditions. The results listed in Table 1 were all modified by their control. There are eight products of *P. notoginseng* fermented by thirty strains indicated increasing antibacterial activity. *Aspergillus oryzae* 1.1395 and *Mucor* sp. 1.498 are two more effective transformers. The component should be changed by the microbes' metabolism system.

TABLE 1
ANTIBACTERIAL ACITIVITY OF P. NOTOGINSENG EXTRACTS FERMENTED BY SELECTED FUNGI

Extracts	*S. flexneri*	*S. aureus*	*E. coli*	*P. aeruginosa*
Aspergillus oryzae 1.1395	2.0	2.4	-	1.4
Mucor sp. 1.805	-	1.2	-	-
Aspergillus sp. 1.44	1.5	-	1.7	-
Mucor sp. 1.806	-	1.3	-	-
Aspergillus niger 1.46	-	-	-	-
Mucor sp. 1.498	1.5	1.0	-	1.3
Aspergillus sp 1.926	-	1.5	-	-

3.2 Cytotoxicity of P. notoginseng extracts fermented by selected fungi

As *P. notoginseng* has 69.46% original activity against the human leukemia cell K562 and the human lung carcinoma cell A549, only extract fermented by *M. rubber* 3081 performed stronger activity at 78.57%. At the same time, almost each fungus culture has no obvious activity. And all tested materials have no activities to A549, although extract fermented by *M. rubber* 3081 got a higher result than its control. The details were listed in Table 2.

TABLE 2
CYTOTOXICITY OF P. NOTOGINSENG EXTRACTS FERMENTED BY SELECTED FUNGI

Sample	K562 %	A549 %
1) DMSO control	1.98	0
DDP control 0.1ug/ml	32.00	0.87
1.0ug/ml	41.41	34.17
10ug/ml	100.00	100.00
P. notoginseng control	69.46	1.16
C. luxiensis 1358 control	48.65	0
Extract fermented by *C. luxiensis* 1358	27.43	0
Paecilomgces hepialli L4 control	53.83	0
Extract fermented by *P. hepialli* L4	53.00	0
Ganoderma lucidum 13 control	17.08	0
Extract fermented by *G. lucidum* 13	67.49	0
M. rubber 3081 control	2.90	10.26
Extract fermented by *M. rubber* 3081	78.57	22.71

3.3 Inhibition against GST-CDC25 phosphatase

Eighteen of thirty cultures showed obvious activity against GST-CDC25 phosphatase. GST-CDC25 phosphatase is the potential target to control tumor replication. The Cdc25 phosphatase's function is as key regulator of the cell cycle during normal eukaryotic cell division and as mediator of the checkpoint response in cells with DNA damage. The role of Cdc25s in cancer has become increasingly evident in recent years. An ideal rate of inhibition for this model is between 80%-90%. Rates in Table 2 indicted that our results are strong.

TABLE 3
INHIBITION AGAINST GST-CDC25 PHOSPHATASE

Extracts	cdc25a %		cdc25b %	
Paraniesslia sp.1.1280	86.41	82.22	92.60	92.10
Aspergillus oryzae 1.1395	85.40	84.10	72.92	70.30
Mucor sp.1.894	83.70	81.40	71.66	71.84
Monascus ruber 3081	79.70	79.40	64.50	62.01
Ophiobolus sp.1.1270	78.92	76.86	86.40	89.15
Paraniesslia sp. 1.1277	77.68	75.40	88.29	92.28
Paraniesslia sp.1.1279	69.15	72.30	83.15	85.18
*Aspergillus niger*1.46	79.00	75.60	84.55	84.43
Aspergillus sp.1.926	68.40	73.80	89.62	87.16
Aspergillus sp.1.927	70.00	66.50	88.34	81.68
Mucor sp.1.806	81.70	80.50	76.21	69.54
Mucor sp.1.713	77.60	80.80	79.62	77.55
Aspergillus sp.1.44	77.80	77.50	81.36	76.32
Aspergillus sp.1.874	79.20	74.00	53.98	51.60
Aspergillus sp.1.495	72.10	66.50	83.10	79.50
Mucor sp.1.805	73.00	75.60	74.12	66.58
Mucor sp.1.1381	75.30	72.80	86.21	81.25
Mucor sp.1.498	66.40	75.20	74.51	79.54

3.4 Difference in the transformation profile

We analyzed chemical components in our extracts by HPLC. There were distinct differences comparing profiles between the extract of unfermented and fermented *P. notoginseng*. In Figure 1, extract of *P. notoginseng* has fewer components than extract of *P. notoginseng* fermented by *Monascus ruber* 3081. There were new chemicals produced in the fermentation process.

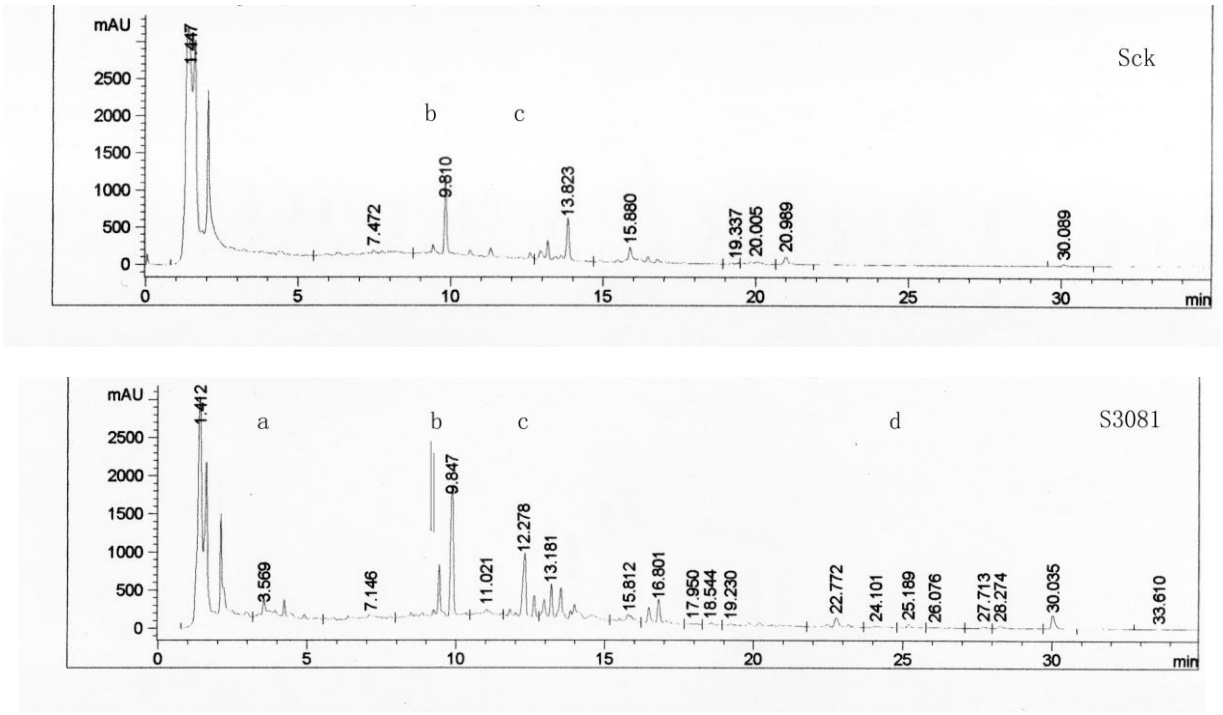

FIGURE 1. DIFFERENCE BETWEEN P. NOTOGINSENG AND FERMENTED P. NOTOGINSENG
Sck: Extract of *P. notoginseng*; S3081: Extract of *P. notoginseng* fermented by *Monascus ruber* 3081.

IV. CONCLUSION

Microbes have significant ability in transformation. Enzymatically-based transformations belong in the standard toolbox of the organic chemist. As many techniques and biocatalysts are now readily available, the field continues to grow steadily by means of many new and exciting developments that have taken place in the past few years. In our study, we tried to use these skills to transform *P. notoginsneg*, a famous Chinese herb. The results proved that it is a useful way to explore new functions of traditional herbs.

Comparing with unfermented *P. notoginseng*, antibacterial and antitumor activity was produced. Because different species have different enzyme systems, it can explain only parts of why transforming products have changes in activity. During the fermented process of *P. notoginseng*, there are various situations. One is that new active metabolites were produced in fungi metabolism, in which components of *P. notoginseng* work as formers. The other reason is that trace active compounds in *P. notoginseng* are accumulated by fungus in the fermenting process, so obviously activity is presented. The third is that components of *P. notoginseng* act as inducers leading to an uncommon enzyme system start up, then new metabolites and new activity was emerged. In fact, our results illustrated in Figure 1 proved that some new components were produced after fermentation. But such hypothesizes need to be proved by further study. We need to determine what indeed happened between fungi and *P. notoginseng*.

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