Annals of Microbiology, **56 (2)** 151-153 (2006)

Production of saponin in fermentation process of Sanchi (*Panax notoginseng*) and biotransformation of saponin by *Bacillus subtilis*

Guo Hong LI[1], Yue Mao SHEN[2], Yajun LIU[1], Ke Qin ZHANG[1]*

[1]Laboratory for Conservation and Utilization of Bio-resource, Yunnan University, Kunming 650091; [2]The State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650204, China

Received 6 September 2005 / Accepted 27 January 2006

Abstract - An antitumor compound ginsenoside Rh_4 was produced during the fermentation process of Sanchi (*Panax notoginseng*) by *Bacillus subtilis*. Saponins ginsenosides Rh_1 and Re were transformed by *B. subtilis* and were produced two main transformed products. The transformed product of ginsenoside Rh_1 was determined to be 3-*O*-β-D-glucopyranosyl-6-*O*-β-D-glucopyranosyl-20(*S*)-protopanaxatriol, and the transformed product of ginsenoside Re had 162 atomic mass units (amu) greater than the substrate. Compared with the substrates, the transformed products had one more glucosyl moiety linked respectively, which indicated that glycosylation reaction occurred in the transformation process.

Key words: fermentation, Sanchi, biotransformation, saponin, *Bacillus subtilis,* glycosylation.

INTRODUCTION

Traditional Chinese Medicine (TCM) as an integral part of Chinese culture has been studied for the effective utilization all along. The fermentation research of TCM started since 80's last century, but only aimed at fermentation of officinal fungi (Wu *et al.*, 2001). Microorganisms have strong ability of transforming substances and the physiological activities of some TCM's substances have been changed by the transformation of intestinal microbes (Zhao *et al.*, 1998), so the microbial fermentation of TCM's substances may produce new compounds or change the content of active components. There is no report on the componential change between TCM's substances and their fermented products.

Sanchi (*Panax notoginseng*) is a famous traditional herb, which has been clinically tested to have the abilities of promoting blood circulation, preventing the formation of blood clots (anti-thrombosis property), dissolving blood clots, enhancing the removal of cellular breakdown products and other debris from the blood circulation. Saponins as the major components in Sanchi have been proved to have diversiform biological activities (Jiang and Qian, 1995; Li and Chu, 1999). Some work related with the transformation and metabolism of Sanchi saponins (Chen *et al.*, 1999; Bae *et al.*, 2000; Lin *et al.*, 2001; Dong *et al.*, 2003) has been done to elucidate that the metabolism of ginsenoside Rb_1 begins with cleavage of the terminal sugar moiety, then gradually the other sugars. The transformation of ginsenoside Rb_1 by *Curvularia lunata* gives two key metabolites, one of which is a new compound. Up to now, there is no report about the microbial fermentation of Sanchi and the glycosylation reaction in the transformation process of saponins. The difference of saponins between original and fermented Sanchi as well as the glycosylation reaction in the transformation process of saponin by *Bacillus subtilis* will be discussed in this paper, by which we hope to provide a new approach to exploit TCM effectively.

MATERIALS AND METHODS

Microbial strain and Sanchi. The strain of *Bacillus subtilis* was purchased from China General Microbiological Culture Collection Center (CGMCC No. 1318) and was conserved in nutrient agar medium.

Sanchi was purchased from medicinal materials market of Yunnan Province, People's Republic of China. Ginsenosides Rh_1 and Re were prepared from Sanchi with method described by Zhou *et al.* (1981).

Fermentation of Sanchi. Sanchi was fermented by *B. subtilis* in solid form. The fibres of Sanchi were ground into powder and mixed with water; the mixture (400 g) was put into flask (1 litre) and sterilized at 121 °C for 40 min. *Bacillus subtilis* (40 ml), cultured in Wort medium, pH 7.0, at 30 °C for 72 h on an orbital shaker at 120 rev min^{-1}, was inoculated into each flask. After culturing at 30 °C for 10 days, the fermented products of Sanchi were dried in oven at 60 °C.

Biotransformation procedures. *Bacillus subtilis* was inoculated in 250 ml flask containing 100 ml Wort medium at pH 7.0. Ginsenoside Rh_1 and ginsenoside Re, dissolved in

* Corresponding author. Phone: + 86 871 5034878;
Fax: +86 871 5034838; E-mail: Kqzhang111@yahoo.com.cn

ethanol, were each added into three flasks of Wort medium inoculated with *B. subtilis* to make the final concentration of ginsenosides Rh_1 0.4 mg/ml and ginsenoside Re 0.2 mg/ml. Two controls of equal volume of ethanol added into the flasks of Wort medium with and without *B. subtilis* were prepared. All the flasks were cultured at 30 °C for 96 h at 120 rev min^{-1}.

Isolation and identification compounds. The fermentation mixture (2 kg) was extracted three times with 80% ethanol exhaustively and dissolved in water; then was extracted five times with *n*-butanol to give 61 g of residue. The residue was subjected over silica gel column (chloroform:methanol:water, 65:35:10, v/v/v), reversed-phase C_{18} (RP-18) column (methanol:water, 65:35, v/v) and Sephadex LH-20 column (methanol) to give compound 1 (16 mg).

The three flasks (300 ml) of *B. subtilis* culture, transforming ginsenoside Rh_1 and Re respectively, were filtered. The filtrates were extracted four times with *n*-butanol, 4.5 g residue containing transformational product of ginsenoside Rh_1 (TRh) and 1.5 g residue containing transformational product of ginsenoside Re (TRe) were obtained, respectively. The fraction TRh was chromatographied on a silica gel column (12 g) eluting with chloroform and methanol (6:1, v/v) and further subjected on Sephadex LH-20 eluting with methanol to obtain compound 2 (5 mg). The fraction TRe was isolated by silica gel column (12 g) and eluted with chloroform, methanol and water (65:35:10, v/v/v), then further purified on Sephadex LH-20 eluting with methanol to obtain compound 3 (1 mg).

Structure of the compounds. Optical rotations were measured on a JASCO DIP-370 Digital Polarimeter. The IR spectra were measured on a Perkin-Elmer-577 spectrophotometer. The NMR spectra were recorded on Bruker AM-400 and Brucker DRX-500 spectrometers. MS were performed on a VG AutoSpec-3000 spectrometer.

Compound 1: white powder, negative FAB-MS *m/z*: 619 ([M – H]$^-$, 100), 600 ([M – H – 18]$^-$, 20); ^{1}H-NMR (C_5D_5N, 400 MHz): 0.83 (3H, s, H-19), 1.02 (3H, s, H-18), 1.22 (3H, s, H-28), 1.57 (6H, s, H-26, H-27), 1.62 (3H, s, H-21), 2.03 (3H, s, H-29), 2.77 (3H, m, H-23), 3.92 (1H, dd, J_1=J_2= 8 Hz, H-2), 4.23 (1H, m, H-6), 4.97 (1H, d, J = 7.7 Hz, H-6-glc-1′), 4.99 (1H, d, J = 7.4 Hz, H-24), 5.47 (1H, d, J = 7.4 Hz, H-22); ^{13}C-NMR (C_5D_5N, 100 MHz): 79.7 (C-3), 78.2 (C-6), 30.5 (C-13,), 140.2 (C-20), 123.6 (C-22), 124.0 (C-24), 106.1 (C-1′), 63.1 (C-6′).

Compound 2: white power, $[\alpha]^{22,D}$ 180 (*c*, 0.1, CH_3OH), negative FAB-MS *m/z*: 799 ([M – H]$^-$, 100), 637 ([M – H – 162]$^-$, 6), 475([M – H – 2×162]$^-$14); IR (KBr) ν 3414, 2958, 1633, 1455, 1146, 1077, 1031 cm^{-1}; ^{1}H-NMR (C_5D_5N, 400 MHz): 1.05 (3H, s, H-19), 1.16 (3H, s, H-18), 4.23 (1H, dd, 9.0, 15.2, H-3), 4.36 (1H, m, H-6), 5.32 (1H, m, H-24), 4.94 (1H, d, 7.7, H-6-glc-1′), 5.91 (1H, d, 8.0, H-6-glc-1″); ^{13}C-NMR (C_5D_5N, 100 MHz): 39.4 (C-1), 27.9 (C-2), 89.2 (C-3), 40.3 (C-4), 61.5 (C-5), 80.3 (C-6), 45.3 (C-7), 41.2 (C-8), 50.2 (C-9), 39.7 (C-10), 32.1 (C-11), 71.1 (C-12), 48.3 (C-13), 51.7 (C-14), 31.3 (C-15), 26.9 (C-16), 54.8 (C-17), 17.4 (C-18), 17.7 (C-19), 73.0 (C-20), 27.1 (C-21), 35.9 (C-22), 23.0 (C-23), 126.4 (C-24), 130.8 (C-25), 25.8 (C-26), 17.6 (C-27), 31.7 (C-28), 16.3 (C-29), 16.8 (C-30), 105.8 (C-1′), 75.6 (C-2′), 80.3 (C-3′), 72.0 (C-4′), 78.6 (C-5′), 62.5 (C-6′), 103.1 (C-1″), 74.8 (C-2″), 77.6 (C-3″), 71.0 (C-4″), 77.6 (C-5″), 62.6 (C-6″).

Compound 3: white power, negative FAB-MS *m/z*: 1108 ([M – H]$^-$, 100), 962 ([M – H – 146]$^-$, 6), 946 ([M – H – 162]$^-$, 6), 476 ([M – 146 – 3×162]$^-$, 11).

RESULTS AND DISCUSSION

According to TLC result, the spot appeared in fermented Sanchi but not in original Sanchi was selected as isolation object, and compound 1 was obtained. From the broths of *B. subtilis* transforming ginsenoside Rh_1 and Re, compounds 2 and 3 were isolated as transformational products respectively.

According to spectra data compound 1 was determined to be ginsenoside Rh_4 (Fig. 1) and, based on the reference of Baek *et al.* (1996), it had cytotoxinic effect to some types of tumor cell. There was no report before on the isolation of ginsenoside Rh_4 from Sanchi that, in our experiment, was not detected by TLC in the extract of unfermented Sanchi, so it must be produced during the fermentation process of Sanchi by *B. subtilis*.

Compound 2 was determined to be 3-*O*-β-D-glucopyranosyl-6-*O*-β-D-glucopyranosyl-20(*S*)-protopanaxatriol (Fig. 2), which was identified as a new compound (Li *et al.*, 2005) and had additional β-glucopyranosyl linked with C-3 compared with the substrate ginsenoside Rh_1 (Fig. 2). Compound 3 gave a quasi-molecular ion peek at *m/z* 1108 ([M – H]$^-$ in the negative FAB-MS, which had 162 atomic mass units (amu) greater than its substrate ginsenoside Re, and indicated that glycosylation replacement reaction had also taken place. Compounds 2 and 3 had higher polarity than the substrates and were not detected by TLC analysis either in other controls or in the methanol extracts of bacterial cell.

Biotransformation was an efficient way to produce new structure products, e.g. the metabolism of ginsenoside Rb_1 by bacteria gave a few novel compounds, but most results showed that some substituents were cleaved from substrates in the transformational process (Chen *et al.*, 1999; Bae *et al.*, 2000). Although glycosylation reaction was general in organism, it was very important in drug research

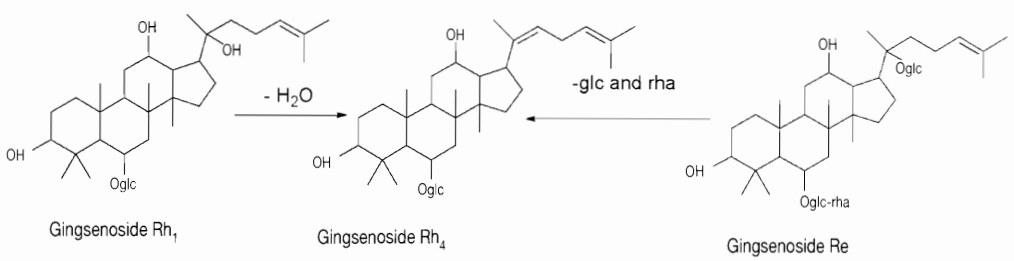

FIG. 1 – The hypothetic pathways of producing ginsenoside Rh_4.

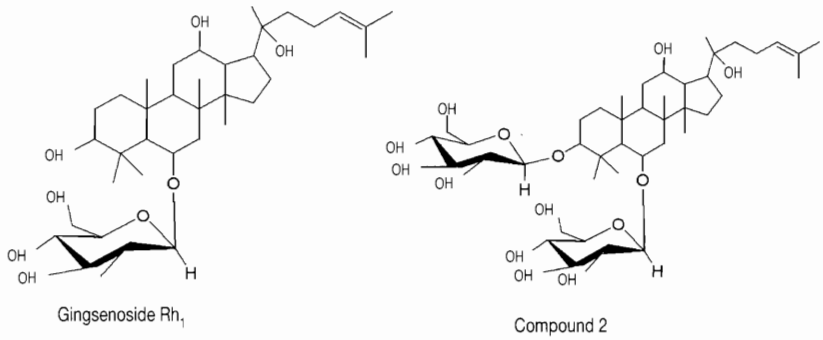

FIG. 2 – The structures of ginsenoside Rh_1 and its transformed product compound 2.

because glycosylation could change polarity of substrate and modulate the metabolism and distribution of active compounds by enhancing the hepatocyte uptake.

Sanchi (*Panax notoginseng*), a famous traditional herb in Southeast Asia, has been widely used, but the Sanchi saponins are not reported on possessing obvious antitumor activity. Based on our study, an antitumor compound ginsenoside Rh_4 was produced in the fermentation process of Sanchi by *B. subtilis,* which proved that microbe could change the components of TCM's substances and resulted in the change of drug effect. In order to investigate the origin of ginsenoside Rh_4 in fermented Sanchi, ginsenosides Rh_1 and Re that had the similar structure with ginsenoside Rh_4 were selected as the objects to study the biotransformation of saponins by *B. subtilis*. The pathways of producing ginsenoside Rh_4 were assumed to be (i) losing hydroxy from ginsenoside Rh_1 or (ii) losing sugar moiety from ginsenoside Re in the transformation process (Fig. 1). But our experiment results showed that glycosylation reaction occurred in the culture of *B. subtilis* after feeding singular compound ginsenoside Rh_1 and Re respectively which was contrary to the hypothesis, so we could not logically explain the origin of ginsenoside Rh_4 in fermented Sanchi.

These results indicate that the fermentation process of TCM's substances by microbes is so complex; however, it might provide diversiform fermented products for screening active compounds. From our experiments it is obvious that the components and drug effect of TCM's substances may be changed after fermentation by microbe and it is feasible to utilize the microbial fermentation of TCM's substances in the discovery of leads and improvement the drug action of TCM.

Acknowledgments

This work was supported by the National Natural Science Foundation (20362010) and Department of Science and Technology of Yunnan Province (2001ZCBFB01C), and we are grateful to Mr. Y.N. He and Ms. H.L. Liang in the Kunming Institute of Botany, Chinese Academy of Sciences, for measuring NMR and MS data, respectively.

REFERENCES

Bae E.A., Park S.Y., Kim D.H. (2000). Constitutive β-glucosidases hydrolyzing ginsenoside Rb_1 and Rb_2 from human intestinal bacterial. Biol. Pharm. Bull., 23: 1481-1485.

Baek N.I., Kim D.S., Lee Y.H., Park J.D., Lee C.B., Kim S.I. (1996). Ginsenoside Rh_4, a genuine dammarane glycoside from Korean red gingseng. Planta Med., 62: 86-87.

Chen X., Zhou Q.L., Wang B.X. (1999). The metabolism of ginsenoside Rb_1 by intestinal bacterial. Acta Pharmacol. Sin., 49: 442-446.

Dong A.L., Ye M., Guo H.Z., Zheng J.H., Guo D.A. (2003). Microbial transformation of ginsenoside Rb_1 by *Rhizopus stolonifer* and *Curvularia lunata*. Biotechnol. Lett., 25: 339-344.

Jiang K.Y., Qian Z.N. (1995). Effects of *Panax notoginseng* saponins on posthypoxic cell damage of neurons *in vitro*. Acta Pharmacol. Sin., 16: 399-402.

Li S.H., Chu Y. (1999). Anti-inflammatory effects of total saponins of *Panax notoginseng*. Acta Pharmacol. Sin., 20: 551-554.

Li G.H., Shen Y.M., Zhang K.Q. (2005). A new saponin transformed from ginsenoside Rh_1 by *Bacillus subtilis*. Chinese Chem. Lett., 16 (3): 359-361.

Lin M.C., Wang K.C., Lee S.S. (2001). Transformation of ginsenosides Rg_1 and Rb_1, and crude Sanchi saponins by human intestinal microflora. J. Chin. Chem. Soc., 48: 113-120.

Wu B.X., Niu J.J., Sun X.L., Dong L.S. (2001). Technology of traditional Chinese medicine's fermentation pharmacy. Shandong Journal of Traditional Chinese Medicine, 20 (3): 179-180.

Zhao R., Xing Z.T., Li X.G., Cheng K.D., Yang X.W. (1998). Chemical modification of natural rug by intestine bacteria. Journal of Jilin Agricultural University, 20 (2): 103-110.

Zhou J., Wu M.Z., Taniyasu S. (1981). Dammarane-Saponins of sanqi-ginseng, roots of *Panax notoginseng*: structures of new saponins, notoginsenosides–R1 and –R2, and identification of ginsenoside-Rg2 and Rh1. Chem. Pharm. Bull., 29: 2844-2850.