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Enzymatic transformation of *Panax notoginseng* stem and leaves for preparation anti-tumor activity constituents

Bin-Hui Jiang[1]*, Yu-Qing Zhao[2]*, Jia-Jun Sun[3], Yan Zhao[1], Xiao-Min Hu[1], Li Liu[1]

[1]College of Resources and Civil Engineering, Northeastern University, Shenyang, 110004, (CHINA)

[2]Shenyang Pharmaceutical University, Shenyang, 110016, (CHINA)

[3]Beijing Thornton Environmental Engineering Co., LTD, Beijing, 101102, (CHINA)

E-mail : jiangbinhui@mail.neu.edu.cn;zhaoyuqingtcm@163.com

ABSTRACT

Ginsenoside bioactive compounds, namely, 20(S)-protopanaxadiol-20-O-β-D-glucopyranoside (Compound-K, C-K) and 20(S)-protopanaxadiol-20-O-β-D-glucopyranoside (compound Mc, G-Mc) were converted from the saponins separated from the stem and leaves wastes of *P. notoginseng* (PNLS) by the industrial enzyme. The enzymatic transformed products C-K and G-Mc were extracted by D-101 macroreticular resins, separated and purified by column chromatographies on silica gel and thin layer gel, and their structures were confirmed by 1H and 13C NMR analysis. Under the optimum enzymatic transformation conditions of pH5.4, 55°C, 20mg $mL^{-1}$of PNLS and 10% (v/v) of ß-glucanase, 88.30mg g^{-1} C-K and 10.21 mg g^{-1} G-Mc was produced after 48 h. © 2014 Trade Science Inc. - INDIA

KEYWORDS

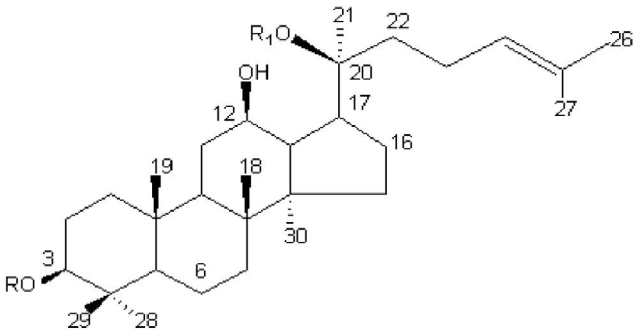
Enzymatic transformation;
Stem and leaves of *Panax notoginseng* (Burk.) F. H. Chen;
Plant saponins;
Compound-K.

INTRODUCTION

Panax notoginseng (Burk.) F. H. Chen, a famous traditional Chinese herbal medicine, has been successful planted artificially about 514×104 hm^2 per year in Yunnan province of China. There are over 1500 t of stem and leaves of *P. notoginseng* were turned out every year, but less than 5% stem and leaves was to be used only, which is raising greatly environment pollution and wasting of resources[1]. It is very important for research on the biotransformation (or bioconversion) of the stem and leaves of *P. notoginseng* by enzymes and microorganisms. Enzymatic transformation, an important component of the biotransformation, is defined as an enzymatic reaction catalyzed by the enzymes from the metabolites of microorganisms, which modify the structures of specific substrates. It has such advantages over chemical synthesis as high stereo-and regio-selectivity, as well as mild reaction conditions, simple operation procedures, less cost and lower pollution, has been used in many fields such as industry, agriculture, medicine and food production, and it would be used widely in the future[2].

Now, it has received wider attention and some researchers found the same pharmaceutical effects between the saponins separated from *P. notoginseng* leaves (*PNLS*) and from *P. notoginseng* roots (*PNRS*), and with lower toxicity[1,2]. Recent epidemiological studies have identified an association between saponins intake and decreased incidence and growth of tumors. The main components in *PNLS* are 20(S)-protopanaxadiol group saponins (ginsenosideRb_1, Rb_2,

Rb_3, Rc, Rd and Rg_3), which are metabolized to C-K, G-Mc, C-Mx, $Rh_2$and PPD (Figure 1) by intestinal microflora of humans or rats, microorganisms and enzymes, exhibited excellent antitumor activities and were responsible for the main pharmacological activities of Ginseng[3-9]. The minor ginsenosides (Rg_3, F_2, C-Y, C-Mx, G-Mc, C-K, Rh_2, and PPD), which are present at low concentrations or absent in wild ginseng, can be produced by hydrolyzing the sugar moieties of the major ginsenosides (Rb_1, Rb_2, Rb_3, Rc, Rd, and Notoginsenoside-Fe, et al.), which comprise more than 80% of the total ginsenosides in wild ginseng[10]. The absorption of the glycosylated major ginsenosides, including Rb_1, Rb_2, and Rc, by the gastrointestinal tract is quite poor[11]. In contrast, the minor ginsenosides as deglycosylated ginsenosides are more readily absorbed into the bloodstream and function as active compounds[12]. As a result, the minor ginsenosides have been demonstrated to be pharmaceutically active and may be excellent potential drug candidates, with activities superior to those of the major ginsenosides[13].



Ginsenoside	R	R_1
Rb_1	O-Glc^{2}-^{1}Glc	O-Glc^{6}-^{1}Glc
Rb_2	O-Glc^{2}-^{1}Glc	O-Glc^{6}-^{1}Arap
Rb_3	O-Glc^{2}-^{1}Glc	O-Glc^{6}-^{1}Xyl
Rc	O-Glc^{2}-^{1}Glc	O-Glc^{6}-^{1}Araf
Rd	O-Glc^{2}-^{1}Glc	O-Glc
Compound O (C-O)	O-Glc	O-Glc^{6}-^{1}Arap
Notoginsenoside-Fe	O-Glc	O-Glc^{6}-^{1}Araf
Rg_3	O-Glc^{2}-^{1}Glc	OH
F_2	O-Glc	O-Glc
Compound Y (C-Y)	OH	O-Glc^{6}-^{1}Arap
Compound Mx (C-Mx)	OH	O-Glc^{6}-^{1} Xyl
Mc	OH	O-Glc^{6}-^{1}Araf
Compound K (C-K)	OH	O-Glc
Rh_2	O-Glc	OH
PPD	OH	OH

Glc, β-D-glucopyranosyl; Arap, a-L-arabinopyranosyl; Xyl, β-D-xylopyranosyl; Araf, a-D-arabinofuranosyl.

Figure 1 : Chemical structures of protopanaxadiol-type ginsenosides

Our previous studies have suggested that a filamentous fungus, *Fusarium sacchari*, was first obtained from the soil-cultivated ginseng, which was verified to possess a potent capacity of transformation of C-K, C-Mx, and G-Mc, and the *in vitro* antitumor activities of C-K, C-Mx, and G-Mc and the *in vivo* antitumor activities of the transformed product mainly containing these compounds were also evaluated[14]. The present works were to explore the hydrolysis of seven saponins by four industrial enzyme preparations, ß-glucanase, cellobiase, glucoamylase and cellulase. And β-glucanase showed the highest activities among the four enzyme preparations to prepare C-K by hydrolyzing the saponin from the stem and leaves of *Panax notogingseng* (Burk.) F.H.Chen (*PNLS*). The enzymatic conversion products were detected and analyzed by HPLC. The chemical structures of which were elucidated with their spectral data of ^{1}H-NMR and ^{13}C-NMR. Two enzymatic transformation products were identified as 20 (S)-protopanaxadiol-20-O-α-L-arabinofuranosyl (1→6)-β-D-glucopyranoside (ginsenoside Mc, G-Mc), and 20 (S)-protopanaxadiol-20-O-β-D-glucopyranoside (C-K), respectively.

MATERIAL AND METHODS

P. notoginseng leaves were obtained from Yunan Province (P. R. China) and identified by Liaoning University of Chinese Traditional Medicine. Other saponins of plant (purity > 80%) were kindly provided by the Research Center of New Medicine of Liaoning University of Chinese Traditional Medicine, they were the leaves saponins of *panax ginseng* PGLS, the total saponins of *panax ginseng* PGTS, the leaves saponins of *American ginseng or panax quinquefolium* PQLS, the fruits saponins of *panax quinquefolium* PQFS, the leaves saponins of *panax notoginseng* PNLS, the total saponins of *panax notoginseng* PNTS, the total saponins of *gynostemma*

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pentaphyllum GPTS. C-K, and G-Mc ($C_{36}H_{62}O_8$, and $C_{41}H_{70}O_{12}$, purity >95%, HPLC) were obtained by silica gel column chromatography and preparative HPLC (C18 column) as described[15]. ß-Glucanase (NS44053, E_1), cellobiase (NS37040, E_2) and glucoamylase (Termamyl CS, E_3) were all provided by Beijing Novey Company. Cellulase (E_4) was bought from Shanghai Lizhu-Dongfeng Biological Technology Co. Ltd, other chemicals were of HPLC grade or the highest quality available. All chemicals and solvents were of analytical or HPLC grade.

Hydrolyzed seven saponins by enzyme

3000g leaves waste of *P. notoginseng* were extracted by alcohol for 24 hours and purified by D_{101} macroreticular resin, 128.6g *PNLS* (purity more than 80%) was obtained. Taking 1mL four enzymes solution each for test groups, and 1mL distilled water as control group, added 1mL 20mg/mL seven saponins samples solution respectively, in the condition of pH4.4, 50°C (25°C only E_3), water bath 48h, then stopped the reaction. The reaction mixtures were extracted three times with BuOH saturated with water and BuOH extracts were evaporated to dryness *in vacuo*, and constant volume with 1mL methanol. The enzymatic hydrolysis of seven saponins were detected by thin-layer chromatography (TLC), and the optimum enzyme for hydrolyzed seven saponins were concluded.

Detection of C-K and G-Mc with HPLC

The essential parts of the HPLC system consisted of a pump, a SPD-10A ultraviolet (UV)-visible detector setting at 203nm, after filtration by Millipore filter of 0.45μm, a 5μL injection loop, a LC workstation of ZHEJIANG university for data collection and a 150mm×4.6mm ID column with Kromasil ODS C_{18}(5μm particle size) stationary phase and a mobile phase of acetonitrile-water (C-K, 60:40; G-Mc, 45:55,v/v) at a flow rate of 1.0mL/min, operated at room temperature.

Preparation of enzymatic transformation products

PNLS (1000mg) was dissolved in AcOH-NaAc buffer solution, pH 5.4. After 30 minutes pre-incubation at 80°C in water bath, the solution was cooled down to room temperature and 50mL ß-glucanase liquid was added to the reactive solution at the final enzymatic concentration of 10 % and the concentration of PNLS 20 mg/mL. After 48 h of incubation at 55 °C in water bath, the solution was cooled down and extracted and purified by D_{101} macroreticular resin with the concentrations of 90%–75% alcohol, the enzymatic transformation products (ETPs, 652.3 mg) were obtained. ETPs were separated by silica gel column chromatography eluting successively with chloroform-methanol-water and finally methanol (8:1→6:1→4:1 →2:1→MeOH by vol). Two main compounds were obtained as T_1 (261.1 mg) and T_2 (180.4 mg) respectively. Their structures were identified according to ^{1}H-NMR and ^{13}C-NMR spectral data.

(a) Kinetic curve of G-Mc by ß-glucanase

G-Mc AcOH-NaAc buffer solution (10 mg/mL, pH 5.4) was added with 20% ß-glucanase liquid. After 5 min, 1h, 2h, 4h, 8h and 24h inoculation at 55°C in water bath, 0.2mL enzymatic reaction solution were collected, extracted and concentrated, and the kinetic curves of G-Mc, and C-K were obtained by the analysis of HPLC, which were performed by Kromasil ODS column (150mm×4.6mm, 5m), room temperature, wave length: 203nm, flow rate: 1mL/min, mobile phase: G-Mc acetonitrile-water (45:55); C-K acetonitrile-water (60:40).

RESULTS AND DISCUSSION

Hydrolytic results of seven samples by four enzyme

After enzymatic hydrolysis, there were changes in seven samples with differently degree. The results of TLC (TABLE 1) showed that there was a new constituent in enzymatic hydrolytic samples (No1~No6) by ß-glucanase and cellulase, which didn't exist in control group, but there were almost not any changes by cellobiase and glucoamylase. The new constituent was produced as compound I. The ability of enzymatic hydrolysis of seven samples by four enzyme was ß-glucanase>cellulose >glucoamylase>cellobiase, and ß-glucanase was the best among them, cellulase was the next.

There was none of changes in No7 after hydrolysis by four enzymes. The principal reason was that the dissolution of No7 was very low in the buffer solution of pH5.4, so the concentration of Jaogulan saponin was inaccessible to that of reaction required. Four enzymes played hardly any role in it. Contrarily, the dissolution of the others was very high in this buffer solution. The results of experiment showed us that the dissolution of samples was one of the important factors in affecting the enzymatic hydrolysis.

According to the results of HPLC, the C-K content of hydrolysis *PNLS* samples by ß-glucanase and cellulase was measured. The results were shown from Figure 2 to Figure 5. Six samples were hydrolyzed by ß-glucanase and cellulase. The ability of enzymatic hydrolytic six samples was No5>No3>No1>No6> No4>No2>>control group for ß-glucanase; No3>No2>No5>No1>No4> No6>> control group for cellulase. The best substratum of ß-glucanase in six saponins was No5 (*PNLS*), the next was No3 (*PQFS*). But the best substratum of cellulase in six saponins was No3 (*PQFS*), the second was No2 (*PGTS*).

TABLE 1 : TLC results of seven enzymatic hydrolysis samples by four enzymes

NO.	Samples	Ability of generating compound I				
		Control group	ß-glucanase	Cellobiase	Glucoamylase	Cellulase
1	PGLS	–	++	–	–	+
2	PGTS	–	+	–	–	++
3	PQFS	–	++	–	–	++
4	PQLS	–	+	–	–	+
5	PNLS	±	+++	±±	+	+
6	PNTS	–	++	–	–	+
7	GPTS	–	–	–	–	–

Note: ±Trace compound I; +Little compound I; ++Some compound I; +++Massive compound I.

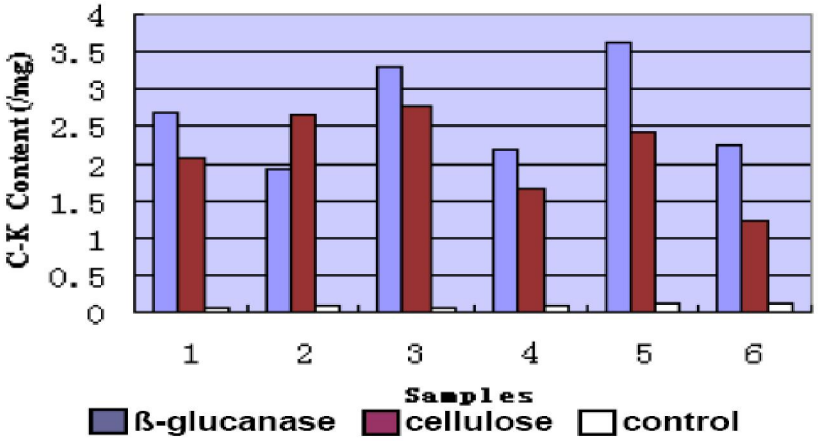

Figure 2 : Change of C-K in samples by enzymatic hydrolysis

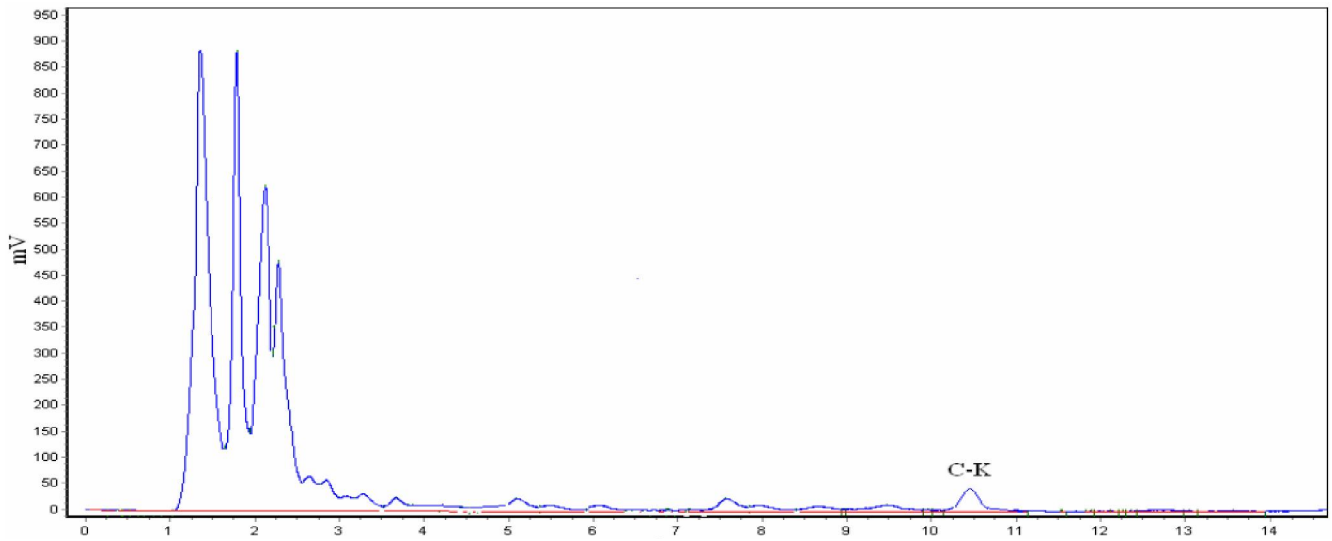

Figure 3 : HPLC of *PNLS*

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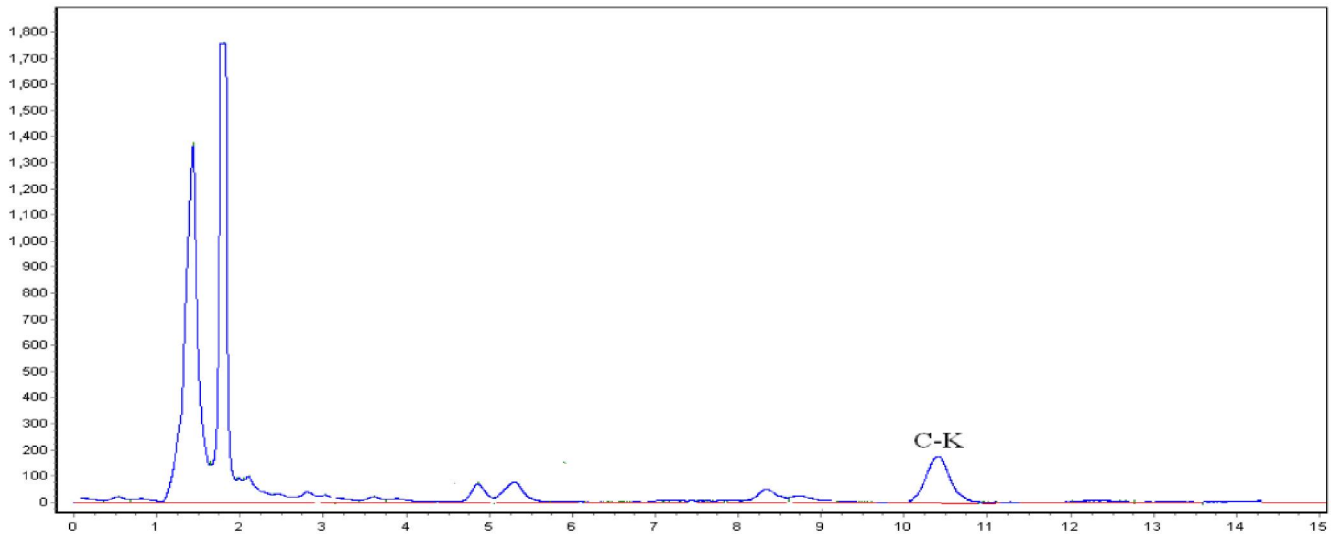


Figure 4 : HPLC of transformed products of *PNLS* by ß-glucanase

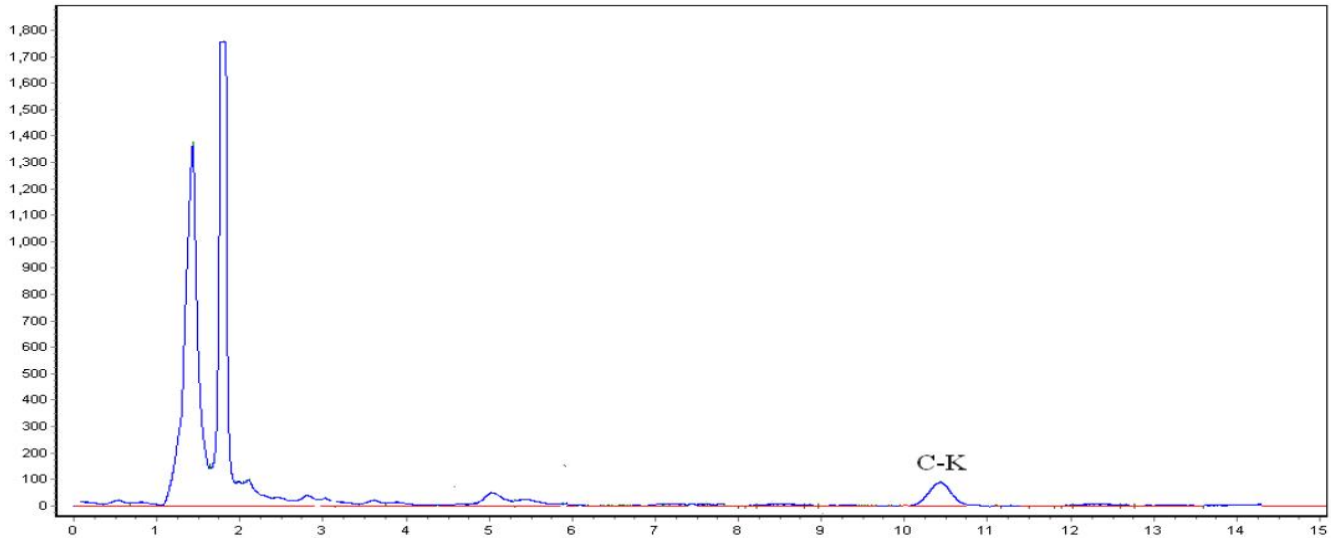


Figure 5 : HPLC of transformed products of *PNLS* by cellulose

The result showed that the higher content of C-K was formed by enzymatic hydrolysis with the samples containing more ginseng protopanaxadiol saponins. As the reported in the previous paper, except of many ginseng protopanaxadiol saponins, such as Rb_1, Rb_3, Rc and Rd, there were also characteristic saponin of *Panax Notoginseng*-Fe in the stem and leaves of *Panax Notoginseng* (Burk.) F.H.Chen[16]. So, the highest content of C-K formed by enzymatic hydrolysis with the leaves of *Panax Notoginseng* (Burk.) F.H.Chen in six saponins.

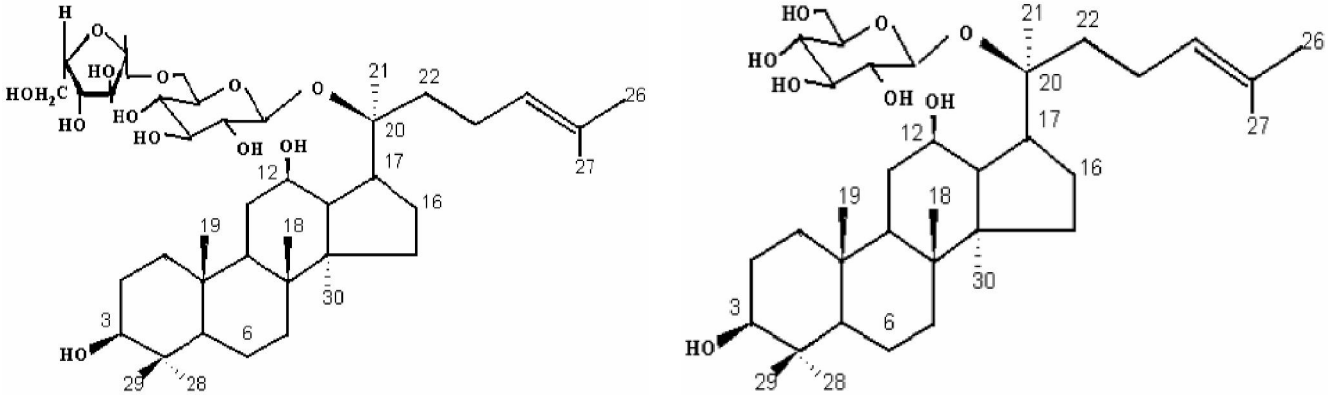
The structure of enzymatic transformed products

T_1

White powder, mp 181–183 °C. ^{1}H NMR(500 MHz pyridine-d_5): δ0.87(3H, s, CH_3-19), 0.92 (3H, s, CH_3-18), 0.98 (3H, s, CH_3-30), 1.02 (3H, s, CH_3-29), 1.21(3H, s, CH_3-28), 1.60(3H, s, CH_3-26), 1.62(3H, s, CH_3-27), 1.65(3H, s, CH_3-21), 5.12(1H, d, J=7.7Hz, H-1'-20-Glc), 5.65(1H, J=1.7Hz, H-1''-6'-Araf). ^{13}C NMR(300 MHz pyridine-d_5): δC 1-30: 39.4, 28.3, 78.1, 39.6, 56.4, 18.8, 35.2, 40.1, 50.3, 37.4, 30.9, 70.3, 49.5, 51.4, 30.9, 26.7, 51.7, 16.3, 16.1, 83.4, 22.4, 36.2, 23.2, 126.1, 131.0, 25.8, 17.9, 28.7, 16.4, 17.5. C-20-Glc C1'-6': 98.1, 75.1, 79.3, 72.2, 76.6, 68.6. C-20-Ara C1''-5'': 110.2, 83.4, 78.9, 86.1, 62.7. It was identified as *20(S)-protopanaxadiol-20-O-α-L-arabinofuranosyl(1→6)-β-D- glucopyranoside* (ginsenoside Mc). (See Figure 6)[17].

T_2

T_2 was obtained as white powder (in.EtOH-H_2O), mp 177–178°C, it could dissolved in Methanol, Ethanol, *n*-BuOH and pyridine, but not in water. ^{1}H NMR(500 MHz pyridine-d_5): δ0.87(3H, s, CH_3-19), 0.91(3H, s, CH_3-18), 0.93(3H, s, CH_3-30), 0.99(3H, s, CH_3-29), 1.58(6H, s, CH_3-26,27), 1.61(3H, s, CH_3-21), 5.19(1H, d, J=7.7 Hz, H-1'-20-Glc).^{13}CNMR (300 MHz pyridine-d_5) : δC1-30 : 39.4, 28.3, 78.3, 39.6, 56.4, 18.8, 35.2, 40.1, 50.3, 37.4, 30.8, 70.2, 49.5, 51.5, 31.0, 26.7, 51.6, 16.4, 16.1, 83.3, 22.4, 36.2, 23.2, 125.9, 130.9, 25.8, 17.8, 28.7, 16.4, 17.4. C-20-Glc C1'-6' : 98.3, 75.2, 79.4, 71.7, 78.1, 62.9. It was confirmed as *20(S)-protopanaxadiol- 20-O-β-D-glucopyranoside*, ginsenoside compound K(C-K), (See Figure 6)[18].



G-Mc C-K

Figure 6 : Chemical structure of enzymatic conversion products

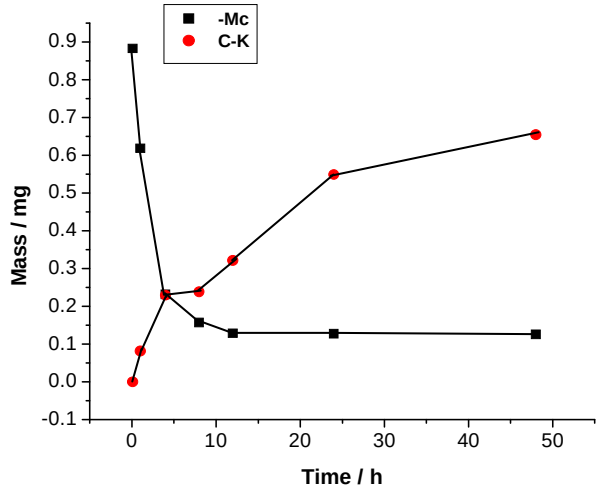


Figure 7 : Kinetic curve of ginsenoside-Mc by ß-glucanase

Transformation kinetic curves

The kinetic curves of G-Mc and C-K were used to elucidate the transformation pathway by ß-glucanase. As seen in Figure 7, the transformation rate of G-Mc to C-K by β-glucanase was over 85 % in 12 h. Besides that, ginsenoside-Rb_1 and-Rb_3 could be transformed to -Rd by ß-glucanase; but no transformation to -Rd, -Rg_2, -Rg_3, or -Rh_2.C-K was one of the main metabolites of ginseng protopanaxadiol saponins by intestinal bacteria. It could be formed through a lot of pathway such as Rb_1 (Rb_3)→Rd→ginsenoside-F_2→C-K; Rb_2→compound I→compound II→C-K and Rc→notoginsenoside-Fe→Mc→C-K. The configuration of arabinosyl differed between compound I and notoginsenoside-Fe, compound II and Mc. Compound I and compound II were pyran-type, notoginsenoside-

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Fe and Mc were furan-type[18-23].

As the pharmacodynamic substructure in natural product, natural active constituents were characterized by lower content, complicated structure and synthesized difficultly. Furthermore, the storage of natural wild resources was decreasing with the development and utilization of plant mendicants. It can promote the extract and separation of natural active constituents, raise the content of effective constituents, and also improve the translation of minor bioactive constituents to apply the enzymatic technology in the field of natural drugs. Because the enzyme had better specificity and its hydrolytic condition was mild, the structures of saccharoid and aglycone were invariable and secondary glycosides could be obtained as hydrolyzing the glycosides. Enzyme hydrolysis could accomplish the reactions that the chemosynthesis couldn't do. Because of lower cost, clearing manufacture, little public hazard and no pollution on environment, it has been played more attention by many scholars.

C-K was unnatural ginsenoside, it was biotransformaed from Rb_1, Rb_2, Rc and Rd. The hydroxyl radical of C_3 was attacked more easily than C_{20} by enzyme, so the product of biotransformation was protopanoxadiol whose 20 hydroxyl radical was bonded with a glucose residue. It was contrary to the effect of acidolysis and alkaline hydrolysis. The acidolysis and alkaline hydrolysis could only break the C_{20} glucosidic bond between the saponin of panaxadiol group and saccharide. Thereby, 20(S, R)-protopanoxadiol, C_{25}-hydroxyl-20 (S, R)-protopanoxadiol and 20(S, R)-panoxadiol were formed by lateral chain isomerization, hydration and cyclization on the acid base condition. Then biotransformation was the only way to get C-K[19]. β- Glucosidase and cellulose were both main enzyme to hydrolyze the saponins of panoxadiol group. They could remove C_3 glucosyl group and C_{20} arabinose group, so that C-K could be gained by changing the saccharide group of protopanoxadiol saponin with the method of enzyme hydrolysis. This established the basis for studying C-K deeply.

CONCLUSIONS

The natural plant could be transformed with the enzymatic technology for preparation the more activity constituents, and raised their contents. When hydrolysis the glycosides bond with enzymes, the conditions were mildness and high specificity. It could keep the structure of activity glycoside invariability and obtain the secondary glycoside. The enzymatic technology was of low cost, no pollution, and simple in manufacturing; so many scholars and enterprises have focused in it.

The result of the present study has indicated that *PNLS* can be transformed into the more activity constituent ginsenoside C-K by ß-glucanase. C-K is one of the major metabolites of ginsenosides, has received increasing attention, because anti-tumor activities in vivo have been shown[6]. But the industrial magnifying and application of C-K has not taken into practice for the limitation of the preparation approach. When the glycosides bond of ginsenosides was hydrolyzed with the industrial enzymes to prepare C-K, the conditions were mildness, low cost, no pollution, and simple in manufacturing and high specificity. Therefore, a novel pathway was provided for the industrial production of C-K in this study.

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