Transformation of Compound K from Saponins in Leaves of *Panax notoginseng* by Immobilized β-Glucanase

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Abstract: **Objective** To prepare an active anti-tumor component, compound K (C-K), from saponins in leaves of *Panax notoginseng* (SLPN) using immobilized β-glucanase. **Methods** Two entrapments, alginate gel-1 (Alg 1) and alginate gel-2 (Alg 2), were evaluated for their ability to immobilize β-glucanase. The amount and purity of C-K obtained from the transformation process were analyzed by HPLC, and the immobilizing parameters were optimized. **Results** β-Glucanase can be immobilized and reused with either of the entrapment. However, using Alg 1 resulted in higher enzyme activity than Alg 2. The optimal concentration of the immobilized enzyme was 10%; The optimal crosslinking time was 4–6 h; and the optimal concentration of the crosslinking agent was 6%–7%. **Conclusion** Immobilized β-glucanase shows sustained enzyme activity, good ethanol tolerance, and was reusable for the preparation of C-K from SLPN.

Key words: β-glucanase; ginsenoside compound K; immobilization; saponins in leaves of *Panax notoginseng* (Burk.) F. H. Chen; transformation

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Introduction

Panax notoginseng (Burk.) F. H. Chen (Araliaceae) is a well known traditional Chinese herbal medicine. Wan (Wan *et al*, 2006) pointed out that *P. notoginseng* showed effects in promoting blood circulation, removing blood stasis, reducing blood clotting, relieving swelling, and alleviating pain; And its isolated saponins possesd anticarcinogenic and hepatoprotective properties, and exerted protective effects on the cardiovascular and cerebrovascular systems. Currently, the total saponins of *P. notoginseng* are used clinically to treat coronary heart disease, cardiac angina, apoplexy, and atherosclerosis (Li *et al*, 2004). The saponins in leaves of *P. notoginseng* (SLPN), phytochemicals obtained from the stems and leaves of *P. notoginseng*, are considered to have similar pharmacological activities as saponins from the root of *P. notoginseng* (Jiang *et al*, 2004a).

Ginsenoside compound K [(20-*O*-β-*D*-glucopyranosyl- 20(*S*)-protopanaxadiol, C-K)] is one of the intestinal bacteria metabolites of panaxadiol-type saponin monomers, such as ginsenoside Rb_1, ginsenoside Rb_2, ginsenoside Rc, and ginsenoside Rd, which are abundant in SLPN (Koizumi *et al*, 1982; Hasegawa *et al*, 1996; Akao *et al*, 1998). Many studies have shown that the intestinal bacterial metabolites of 20(*S*)-protopanaxadiol-type ginsenosides, like C-K, have various pharmacological activities *in vitro* and *in vivo*, including anti-metastatic or anti-carcinogenic effects by blocking tumor invasion or preventing chromosomal activation and tumorigenesis (Chi and Ji, 2005; He *et al*, 2005), anti-inflammatory, and anti-allergic effects (Shin *et al*, 2005), reduction of doxorubicin toxicity in mouse testis, and hypothalamo-pituitary-adrenal axis-modulating activity (Paek *et al*, 2006). Additionally, C-K plays an important role in the pathophysiology of neurodegenerative disease and in protecting the central

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nervous system (Jang *et al*, 2004). C-K has been attracting the increasing attention because the *in vivo* anti-metastatic and anti-allergic activities of the ginsenosides have been shown to be mediated by this metabolite (Kim *et al*, 2004). However, the practical application of such a powerful anti-tumor compound is limited due to its low content in SLPN (Jiang *et al*, 2004b). Better extraction and purification methods are necessary in order to obtain sufficient quantities of C-K.

β-Glucanase has all the enzyme activities of β-glucase, cellulase, and hemicellulase. It can hydrolyze steroid compounds into saponions with less glycoside effectively, because it can break the β-glycoside bonds exclusively. According to Chi (Chi and Ji, 2005), the transformation pathway of ginsenoside by β-glucanase can be proposed in Fig. 1. In our previous work, transformation of SLPN by free β-glucanase has been studied, and highly concentrated C-K (0.883 mg/mL) was obtained from the process (Jiang *et al*, 2004c).

Immobilization techniques may open the door for more large-scale preparation of limited natural active compounds since immobilization can directly deliver free cells or enzymes to the target location through chemical or physical methods. This technique provides various advantages over the use of free enzymes, including easier recovery of the enzyme, the ability to reuse the enzyme (Varavinit *et al*, 2001), simple operation, improved stability (Ikeda and Kurokawa, 2001), facilitated product separation, continuous operation (Yang *et al*, 2004), and increased tolerance to denaturing conditions. Conventional immobilization methods are generally classified into four categories: covalent binding, chemical cross-linking, adsorption, and entrapment processes. Enzyme immobilization by entrapment using an alginate gel is fast, low-cost and allows the enzyme to retain its native structure. Therefore, it has been used with a wide range of biocatalysts (Ikeda *et al*, 2002).

Immobilization techniques have been widely used in food and wine production, chemical and environmental engineering, and pharmaceutical production. Various enzymes have been used in the immobilization processes, including hemicellulase, trypsase, lipase, and β-glucase. However, no information is available on the immobilization of β-glucanase, and there has not been a report in the literature about using an immobilized enzyme to transform ginsenosides to C-K.

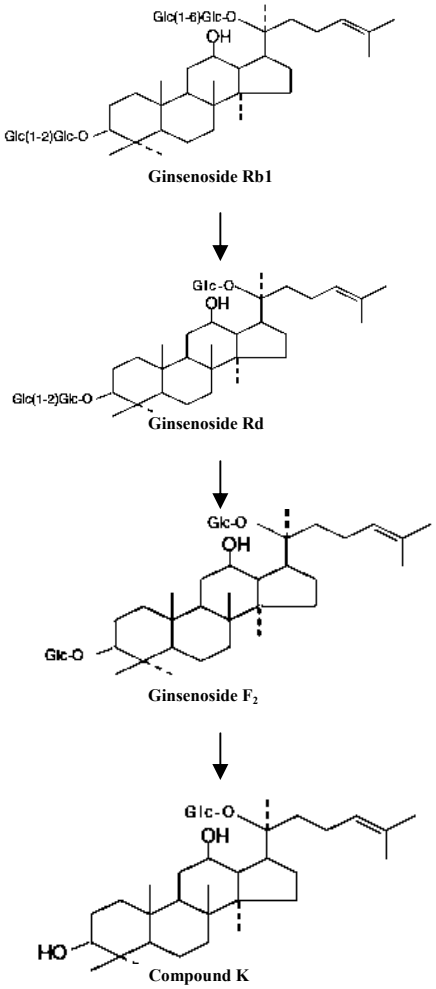

Fig. 1 Proposed transformation pathway of ginsenoside Rg1 to C-K

In the present study, we report the application of immobilized β-glucanase using entrapment to biotransform SLPN to C-K for the first time. The efficiency of immobilized β-glucanase in the transformation process was also evaluated.

Materials and methods

Materials and instruments

SLPN was purchased from Yongchang, Yunnan Province (purity > 80%); β-glucanase (NS44053) was provided by Beijing Nuoweixin Company; Sodium alginate (Na-Alg) with chemical grade was purchased from Tianjin Yuanhang Chemical Co., Ltd.; Chitosan was provided by LI Liang, a lecturer in Northeastern University.

Standard C-K (purity > 90%) was provided by Prof. ZHAO Yu-qing, Liaoning New Medicine Developing Center. Both acetonitrile and methanol were chromatographic grade, other reagents were analytical grade.

A Hitachi L-7100 HPLC system (Japan), equipped with N2000 spectrum workstation (Zhejiang University, China) was utilized; as for an 80-2 centrifuge (Operating Instruments Factory, Shanghai); other instruments, such as an AS-3120A hyperacoustic machine (Tianjin Auto-science Instrument Co., Ltd.) were also used.

Preparation of SLPN solution

LSPN was weighed and dissolved in HAc-NaAc buffer solution (pH 5.5) in a volumetric flask to make a final concentration of 20 mg/mL. The SLPN solution was stored at 4 ℃ for later use.

Preparation of immobilized enzyme

The immobilized enzyme was prepared as described by Wang (Wang, 2002) with some modifications. Two Alginate methods were assessed and the compounding was as following: the gelling agent for both methods was NaAlg in water, 0.03 g/mL; the cross-linking agent for Alg 1 was 2%–7% $CaCl_2$ solution and that for Alg 2 was 5% $CaCl_2$ solution + 1% chitosan HAc solution. An initial solution of NaAlg in water, 0.03 g/mL was obtained by stirring a given amount of Na-Alg with water and melting in a water bath at 90 ℃, forming the gelling agent. After the Na-Alg solution was cooled down to 30–40 ℃, β-glucanase was added and mixed in the solution. The mixture was then extruded dropwise *via* a 5 mL syringe into 50 mL of the cross-linking agent. After the calcium alginate beads with entrapped enzyme were hardened in the cross-linking agent for a period of time at 4 ℃, the beads were taken out and rinsed three times with distilled water, then stored at 4 ℃ for later use.

Preparation of C-K by immobilized enzyme

Immobilized enzyme was prepared according to different immobilization methods for optimization. To each of three groups of 1 mL SLPN solution, 1 mL of 20% β-glucanase (free enzyme), 1 mL of immobilized β-glucanase, and 1 mL of distilled water (control) were added, res-pectively, and incubated for 48 h at 55 ℃ in a water bath. Water-saturated *n*-butanol (1 mL) was added to the reaction mixture, followed by shaking. Then the mixture was centrifuged at 2000 r/min for 5 min and the *n*-butanol layer was transferred to an evaporating dish. Two volumes of *n*-butanol were added to perform the liquid-liquid extraction. The *n*-butanol layer was evaporated to dryness, then dissolved in 2 mL chroma-tographic grade methanol and filtered on a 0.45 μm micropore film before HPLC analysis.

In order to test the ability to reuse the immobilized enzyme, the enzyme remaining in the reaction was reused for a second, third, forth, and even fifth purification, followed by the same procedures described above for further HPLC analysis.

HPLC analysis of C-K released by immobilized enzyme

Chromatographic condition The chromato-graphic column used was a Kromasil ODS column (150 mm × 4.6 mm, 5 μm) (Knauer, German) at room temperature. The detection wavelength was 203 nm; the flow rate was 1 mL/min and the mobile phase was acetonitrile-water (60∶40).

Standard curve and linear range A C-K standard was weighed and dissolved in methanol in a volumetric flask to make a final concentration of 0.54 mg/mL. The methanol solutions (1, 3, 5, 7, 9, and 11 μL) were injected into the HPLC under the analytical conditions described above. The standard curve was drawn with the peak area as the Y-axis and C-K concentration as the X-axis, and excellent linearity in the range of 0.54–7.02 μg (linear regression equation: $Y = 333\ 821.39\ X + 12\ 093.48$; $r = 0.9997$) was observed.

Determination of C-K transformed by immobilized enzyme A 5 μL sample was injected into the HPLC. The peak area of C-K was determined under the chroma-tographic conditions described above. The C-K content was calculated based on the linear regression equation.

Results and discussion

Identification of C-K

Fig. 2 shows the HPLC chromatograms of the C-K standard (A), enzymatic transformation product (B), and control group (C). The formation of C-K from enzymatic transformation was identified. C-K was previously isolated and identified by our colleagues, and it was confirmed that the retention time (t_R) of C-K was

6.598 min in the standard C-K chromatogram shown in Fig. 2 (A), the spectra of the immobilized enzymatic product shown in Fig. 2 (B), in which a peak was observed at 6.598 min, demonstrating the formation of C-K. It was evident that there was almost no C-K presented in the control shown in Fig. 2 (C). These results indicated that considerable amount of C-K, which was naturally present at low levels in SLPN, was produced after transformation of SLPN by immobilized β-glucanase.

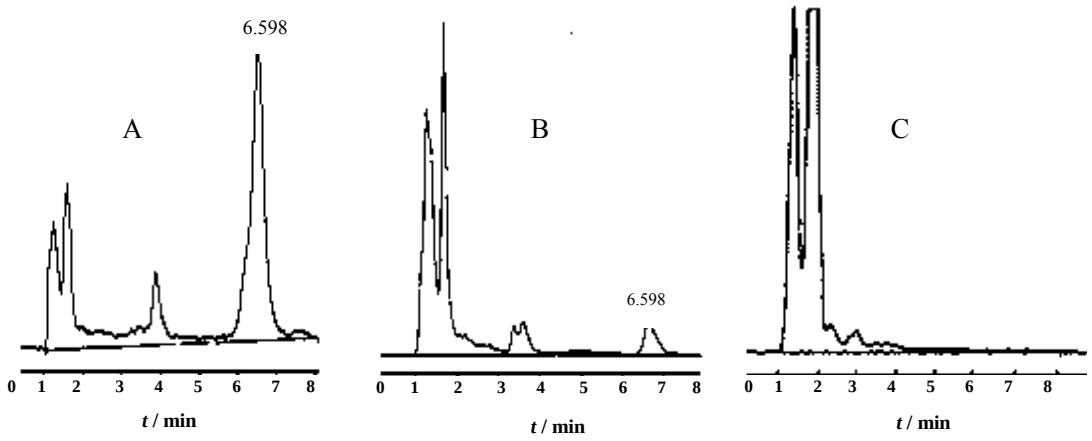

Fig. 2 HPLC Chromatograms of C-K standard (A), transformation product (B), and control (C)

Comparison between the two calcium alginate immobilization methods

Two calcium alginate immobilization methods were compared and summarized in Table 1. As shown in the table, the method using Alg 1 showed higher enzyme activity and transformed more C-K from SLPN than Alg 2. Further studies regarding the use of Alg 1 were accomplished to optimize the methods. The difference between the two methods was that chitosan (polycation) was included in the Alg 2 method, but not in the Alg 1 method. Chitosan can react with alginate (polyanion) through electrostatic interactions to form a polyelectrolyte semi-permeable membrane, which might increase the diffusion resistance of the substrate, and hence decrease the enzyme activity and reduce the amount of C-K transformed from SLPN.

The amount of C-K obtained was not significantly different between samples prepared following 5 and 16 h of cross-linking for Alg 1; but for Alg 2, cross-linking for 5 h was better than 16 h. This may be explained by the immobilization mechanism. Sodium alginate is a linear high polymer, which consists of β-1, 4-*D* mannuronic acid (M) and α-1, 4-*L* guluronic acid (G). There are three arrangements for M and G, —M—M—M—, —G—G—G—, and —M—G—M—G—. Two G molecule chains can easily react with Ca^{2+}, form a hole between them, and result in the irreversible formation of a gel, producing the so-called "egg box" pattern (shown in Fig. 3).

The enzyme is then fixed in the network of the gel

Table 1 Tansformation effect of two calcium alginate immobilization methods

Alg 1 treatment time / h	C-K / ($mg \cdot g^{-1}$)	Alg 2 treatment time / h	C-K / ($mg \cdot g^{-1}$)
5^{1st}	41.51	5^{1st}	20.41
5^{2nd}	63.20	5^{2nd}	32.40
5^{3rd}	59.32	5^{3rd}	40.08
5^{4th}	53.70	5^{4th}	40.30
16^{1st}	38.01	16^{1st}	10.28
16^{2nd}	60.86	16^{2nd}	27.80
16^{3rd}	70.03	16^{3rd}	27.61
16^{4th}	41.33	16^{4th}	17.99

Note: Both 5 h and 16 h meant the cross-linking time of the immobilized enzyme
1^{st}, 2^{nd}, 3^{rd}, and 4^{th} were the reused cycles of immobilized enzyme

Fig. 3 "Egg box" pattern formed by alginate and Ca^{2+}

(Sun *et al*, 2004). The gel reaction would not be complete if the cross-liking time was too short. On the other hand, if the cross-liking time was too long the gel would be too compact, which would increase the diffusion resistance and decrease the enzyme activity, then the amount of C-K produced decreased accordingly. Furthermore, the amount of leaking enzyme would be increased if the cross-linking time was longer. As for Alg 2, longer cross-linking time could make the membrane more compact, and then lower amount of C-K was produced.

The amount of C-K transformed by immobilized enzyme did not vary significantly after four-cycles of reuse for both Alg 1 and Alg 2. Therefore, both calcium alginate immobilizing methods were capable of allowing the enzyme to be reused.

Optimization of immobilized β-glucanase concentration

Table 2 shows the effect of the β-glucanase concentration on immobilization. Enzymes with different concentration, 5%, 10%, 20%, and 30%, were chosen to be entrapped in Na-Alg. As shown in Table 2, reactions with 5% and 10% enzyme resulted in more C-K transformation, and the maximum C-K content reached 60.19 mg/g with 10% β-glucanase in the reaction, demonstrating that the optimal β-glucanase concentration for C-K transformation was 10%. Furthermore, the amount of transformed C-K was comparable after three reuse cycles, which indicated that the immobilized β-glucanase had high reusability.

Optimization of cross-linking time for immobilized β-glucanase

The effect of cross-linking time on immobilized enzyme is presented in Table 3. Different cross-linking time periods (4, 6, 8, 12, and 24 h) were chosen, and the enzyme was reused for three cycles. As shown in Table 3, the cross-linking time had minimal impact on the ability of immobilized enzyme to accomplish C-K transformation, which was consistent with the observation discussed above. The enzyme in the reaction showed higher activity after cross-linking for 4–6 h. Therefore, cross-linking for 4–6 h was selected as the most appropriate for C-K transformation.

Table 2 Effects of enzyme concentration on immobilization

Enzyme concentration / %	C-K / ($mg \cdot g^{-1}$)
5^{1st}	46.12
5^{2nd}	50.99
5^{3rd}	45.68
10^{1st}	60.19
10^{2nd}	58.38
10^{3rd}	41.41
20^{1st}	20.71
20^{2nd}	29.82
20^{3rd}	19.49
30^{1st}	26.96
30^{2nd}	23.18
30^{3rd}	19.36

Table 3 Effects of cross-linking time on immobilization

Cross-linking time / h	C-K / ($mg \cdot g^{-1}$)
4^{1st}	62.27
6^{1st}	70.49
8^{1st}	58.45
12^{1st}	60.82
24^{1st}	61.88
4^{2nd}	59.50
6^{2nd}	70.57
8^{2nd}	55.39
12^{2nd}	53.98
24^{2nd}	48.48
4^{3rd}	56.63
6^{3rd}	55.28
8^{3rd}	42.61
12^{3rd}	50.16
24^{3rd}	43.05

Optimization of cross-linking agent concentration

Na-Alg can be easily cross-linked by reagents like $CaCl_2$. The strength and elasticity of the beads were correlated to the concentration of cross-linking agent. Concentration of $CaCl_2$ from 3% to 7% was chosen for elucidating the effect of $CaCl_2$ on immobilized enzyme activity, and the results are shown in Fig. 4.

It can be noted that as the concentration of $CaCl_2$ increased, the activity of immobilized β-glucanase increased accordingly. However, the amount of transformed C-K remained almost constant when concentration of $CaCl_2$ was up to 6%–7%. So a 6%–7% concentration of the cross-linking agent for immobilized β-glucanase was selected to be used for C-K transformation.

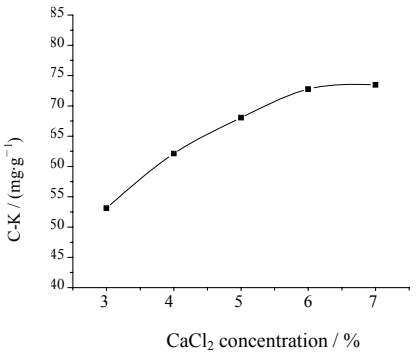

Fig. 4 Effects of $CaCl_2$ concentration on immobilized enzyme

Effect of ethanol on β-glucanase

To determine the tolerance of the immobilized enzyme to denaturing conditions, the effect of different concentration of ethanol on the enzyme was studied, and the results are shown in Fig. 5.

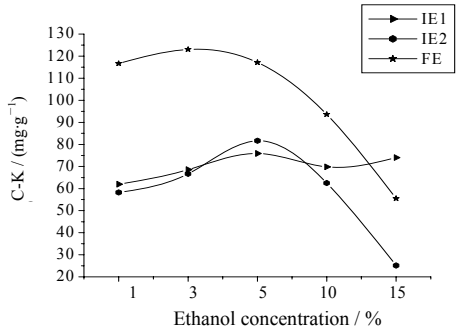

Fig. 5 Effects of ethanol on β-glucanase activity

Note: IE 1 means adding the ethanol to the gelling agent in the process of immobilizing the enzyme before incubation with substrate

IE 2 means adding the ethanol to the substrate before incubation with the immobilized enzyme

FE means adding the ethanol to the substrate before incubation with free enzyme

The activity of free enzyme (FE) reached the highest level when the concentration of ethanol was 3%, and dropped quickly when the concentration of ethanol was greater than 5%. However, the activity of immobilized enzyme (IE) 1 remained stable from ethanol concentration 1% to 15%, indicating that IE 1 had a higher ethanol tolerance than FE. The activity of IE 2 was also increased when the concentration of ethanol was 1% to 5% and decreased when the concentration of ethanol was 5% to 15%. Therefore, the highest enzyme activity of IE 2 was observed when the concentration of ethanol was 5%, also demonstrating higher ethanol tolerance than FE. In sum, the immobilized enzymes, IE1 and IE2, showed better ethanol tolerance than FE; and IE1 showed higher ethanol tolerance than IE2.

Conclusion

The present study reports the application of immobilized β-glucanase entrapped using an alginate gel to generate C-K from SLPN for the first time. Two different cross-linking agents were compared and both exhibited the ability to be reused. However, the enzyme activity using Alg 1 was higher than using Alg 2.

Further optimization of the immobilization parameters revealed that the optimal immobilized enzyme concentration was 10%; The optimal cross-linking time was 4–6 h; And the optimal concentration of cross-linking agent was 6%–7%.

The effect of ethanol on β-glucanase indicated that the free enzyme showed the highest activity when the ethanol concentration was 3%, and the enzyme activity decreased dramatically when the ethanol concentration was greater than 3%. However, the immobilized β-glucanase activity reached a maximum when the concentration of ethanol was 5%, higher than that for the FE (3%), and the enzyme remained active at a concentration up to 15% ethanol. Moreover, the IE exhibited better stability than the FE.

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