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Communication

Optimization of Enzymatic Treatment for Compound K Production from White Ginseng Extract by Response Surface Methodology

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Ginsenoside 20-*O*-*β*-D glucopyranosyl-20(*S*)-protopanaxadiol (compound K), a minor ginsenoside, is not found in white raw ginseng, but has better bioavailability than the major ginsenosides in ginseng. Employing commercial enzyme packages for industrial applications, the optimum conditions for enzymatic transformation for the highest content of compound K was explored to enhance the health benefits of ginseng extract. Cytolase PCL 5 was selected from commercial enzyme packages nominated for high *β*-glucosidase activity. By response surface methodology, the optimal conditions were identified as 78 h of treatment at pH 4.3 at 55.4 °C for 2.068 mg/mL of compound K, showing good agreement with the experimental value.

Key words: ginsenoside; ginsenoside 20-*O*-*β*-D glucopyranosyl-20(*S*)-protopanaxadiol (compound K); enzymatic transformation; optimization; response surface methodology

Ginsenosides are known for various health benefits, including anti-fatigue,[1] anti-hypertensive,[2] anti-allergic,[3] and anti-diabetic activities.[4] In wild ginseng, more than 80% of total ginsenosides are glycosylated major ginsenosides such as Rb1, Rb2, Rc, Rd, Re, and Rg1,[5] but the absorption of those major ginsenosides *via* the gastrointestinal tract is poor.[6] On the contrary, minor ginsenosides (F_1, F_2, Rg3, Rh_1, Rh_2, compound Y, compound Mc, and compound K), which can be produced by hydrolysis of the sugar moieties of major ginsenosides, exhibit better bioactivity than the major ginsenosides due to enhanced bioavailability.[7] Especially compound K, which is not found in raw white ginseng, is of particular interest for broad applications in medicine due to several remarkable biological properties such as anti-tumor,[8] anti-allergic,[9] and hepatoprotective effects.[10] Moreover, studies have found that compound K is the major form of protopanaxadiol-type saponins absorbed in the intestines.[11]

Several approaches to the transformation of major ginsenosides to minor ones have been attempted by mild acid hydrolysis,[12] alkaline cleavage,[13] heating,[14] microbial transformation,[15,16] and enzymatic conversion.[17] Enzymatic hydrolysis allows specific hydrolysis of the glucose moiety from the ginsenoside skeleton, which includes glucose, L-arabinopyranoside, L-arabinofuranoside, D-xylose, and/or L-rhamnose.[18] Thus, *β*-glucosdase,[19] *β*-xylosidase,[20] *α*-L-arabinofuranosidase,[21] and *α*-L-rhamnosidase[22] can be used to hydrolyze ginsenosides. Accordingly, *β*-D-glucosidases and glycosidases, including *β*-D-glycosidase, cellulase, lactase, pectinase, hesperidinase (flavanone *β*-D-glycosidase), and naringinase (flavonoid *β*-D-glycosidase), which are purified from various microorganisms such as *Aspergillus niger*,[23] *Aspergillus oryzae*,[24] *Pencillium* sp.,[25] *Sulfolobus* sp.,[26] and *Thermus caldophilus*,[27] are used to produce diverse minor ginsenosides.

Considering the complexity of the stepwise process by sequential enzymatic transformation, the single-step process employing commercial enzyme packages would be a simpler and more practical method to produce compound K from white ginseng extract for economic reasons. Based on the premise that the use of multiple enzymes should improve the yield of compound K production, here we explored a practical method of producing compound K from raw white ginseng extract using commercial hydrolyzing enzyme packages.

Since pectinases from *Aspergillus niger* are known to exhibit strong hydrolysis activity, and thus have been used in various enzymatic food processes such as debittering of juices and refining wine,[28] several brands of pectinase from *Aspergillus niger*, listed in Table 1, were subjected to screening for the production of compound K. Although conditional factors dictate the yield of compound K by determining the activity of the enzyme, the maximum yield of compound K relies on the characteristics of the enzyme package. To screen enzyme packages, we compared the yield of compound K derived by 60 h of transformation under four sets of optimum conditions that were common to all the enzyme packages tested: pH 4.0 at 55 °C, pH 4.5 at 55 °C, pH 4.0 at 50 °C, and pH 4.5 at 50 °C. White

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Abbreviations: ANOVA, analysis of variance; compound K, ginsenoside 20-*O*-*β*-D glucopyranosyl-20(*S*)-protopanaxadiol; F2, 3-*O*-(*β*-D-glucopyranosyl)-20-*O*-*β*-D-glucopyranosyl-20(*S*)-protopanaxadiol; HPLC, high-performance liquid chromatography; Rb1, 2-*O*-*β*-glucopyranosyl-(3*β*,12*β*)-20-[(6-*O*-*β*-D-glucopyranosyl-*β*-D-glucopyranosyl)oxy]-12-hydroxydammar-24-en-3-yl *β*-D-glucopyranoside; Rb2, 3-*O*-[*β*-D-glucopyranosyl-(1—2)-*β*-D-glucopyranosyl]-20-*O*-[a-L-arabinopyranosyl-(1—6)-*β*-D-glucopyranosyl]-20(*S*)-protopanaxadiol; Rc, 3-*O*-[*β*-D-glucopyranosyl-(1—2)-*β*-D-glucopyranosyl]-20-*O*-[a-L-arabinofuranosyl-(1—6)-*β*-D-glucopyranosyl]-20(*S*)-protopanaxadiol; Rd, 3-*O*-[*β*-D-glucopyranosyl-(1—2)-*β*-D-glucopyranosyl]-20-*O*-*β*-D-glucopyranosyl-20(*S*)-protopanaxadiol; Rf, (3b,6a,12b)-3,12,20-trihydroxydammar-24-en-6-yl-2-*O*-(*β*-D-glucopyranosyl)-b-D-glucopyranoside; Rg3, 3-*O*-[*β*-D-glucopyranosyl-(1→2)-*β*-D-glucopyranosyl]-20(*S*)-protopanaxadiol; RSM, response surface methodology

Table 1. Ginsenoside Compound K (mg/mL) Content of White Ginseng Extract after 60 h of Enzymatic Treatment with Five Different Enzyme Packages

Commercial enzyme	Temperature (°C)	pH	Ginsenoside compound K (mg/mL)
Cytolase PCL5	55	4.5	0.960 ± 0.075
		4.0	0.890 ± 0.049
	50	4.5	0.880 ± 0.058
		4.0	1.142 ± 0.050
Sumizyme AC	55	4.5	0.930 ± 0.004
		4.0	0.918 ± 0.013
	50	4.5	1.156 ± 0.002
		4.0	0.845 ± 0.043
Multifect Pectinase FE	55	4.5	0.249 ± 0.032
		4.0	0.303 ± 0.037
	50	4.5	0.368 ± 0.038
		4.0	0.414 ± 0.033
Crystalzyme PML-MX	55	4.5	none
		4.0	none
	50	4.5	none
		4.0	none
Pectinex Ultra AFP	55	4.5	0.628 ± 0.033
		4.0	0.632 ± 0.028
	50	4.5	0.808 ± 0.084
		4.0	0.747 ± 0.062

ginseng extract was prepared by incubating dried raw ginseng root powders (particle size $\leq 250\,\mu m$) in 90% ethanol for 24 h at 70 °C. Enzymatic transformation was performed by incubation in a shaking water bath after the addition of 1 mg of each enzyme package to 5 mL of prepared ginseng extract. Following the fractionation of extract using butanol, the ginsenosides were quantitatively determined at a wavelength of 203 nm by the HPLC system (600S controller; Waters, Milford, MA) with a C18-bonded reversed-phase silica column (Venusil XBP C18, 4.6 mm × 250 mm, 5 μm; Agela Technologies, Wilmington, DE) performed at a flow rate of 1.6 mL/min of the mobile phase composed of water and acetonitrile.

When the ginesnosides in the white ginseng extract, the concentration of which was adjusted to 7°Brix, were analyzed, only major ginsenosides such as Rb_1 (2.14 mg/mL), Rc (0.9 mg/mL), Rb_2 (1.68 mg/mL), and Rd (0.48 mg/mL) were found. After 60 h of treatment, these ginsenosides were converted to Rg_3, F_2, and compound K, were different from the tested enzyme packages (Table 1). Crystalzyme PML-MX did not transform the ginsenosides into compound K. Sumizyme AC and Cytolase PCL5 showed conspicuous yields of compound K superior to Pectinex Ultra AFP followed by Multifect Pectinase FE under the same conditions. Although the highest yield of compound K (1.156 mg/mL) was achieved using Sumizyme AC at pH 4.5 at 50 °C, Cytolase PCL5 (1.142 mg/mL) showed comparable activity. Hence the transformation activities of Sumizyme AC and Cytolase PCL5 were investigated further *via* 70 h of longer treatment under a wider range of temperatures and pH's. Cytolase PCL5 at pH 4.3 at 50 °C accumulated the highest amount of compound K (1.627 mg/mL). In fact, under most of the conditions imposed except pH 4.3 at 55 °C, Cytolase PCL5 produced more compound K than Sumizyme AC.

Table 2. Analysis of Variance of Independent Variables for Optimization of Compound K Production

Regression	DF[a]	Type I Sum of Squares	F Value	Pr > F
Linear	3	0.06	0.25	0.87
Quadratic	3	2.22	10.28	0.01*
Interaction	3	0.02	0.09	0.96
Total Model	9	2.29	3.54	0.09

[a]Degree of freedom
*Significant at $p < 0.05$

Remembering that the yields of compound K at a reaction time of 60 h were similar, the observed superiority indicates that Cytolase PCL5 is better than Sumizyme AC. Further, the yield of compound K with Cytolase PCL5 increased as the temperature decreased to 50 °C. Hence Cytolase PCL5 was finally selected as the most effective enzyme package among those tested.

The optimum condition for ginsenoside compound K production with Cytolase PCL5 was explored by a response surface methodology (RSM). As control factors, the reaction time (X_1), temperature (X_2) and pH (X_3) were considered based on the results of the preliminary experiments. Since the yield of compound K decreased after 84 h of reaction time, the respective low and high levels of the individual factors were coded as 72 h (−1) and 84 h (+1) for reaction time; 50 °C (−1) and 60 °C (1) for temperature; and 3.6 (−1) and 5.0 (1) for pH. According to the Box-Behnken design, 15 experiments were carried out. The average experimental compound K yield values varied from 0.957 to 2.312 mg/mL. A second-order polynomial regression model (eq. (1)) made possible the prediction of the effects of the three parameters on compound K yield (Y).

$$\begin{aligned} Y = {} & (-144.486) + 1.497X_1 + 2.498X_2 + 8.821X_3 \\ & + (-0.009)X_1^2 + (-0.022)X_2^2 + (-1.046)X_3^2 \\ & + (-0.001)X_1X_2 + (-0.008)X_1X_3 + 0.015X_2X_3 \quad (1) \end{aligned}$$

Analysis of variance (ANOVA) was done to check the statistical significance of the model and the data (Table 2). The coefficient of determination (R^2) of the model was 0.864. The ANOVA results revealed that the quadratic term was significant ($p < 0.05$), while the other terms showed no significance. The linear term of temperature (X_2), and the quadratic terms of temperature (X_2^2) and pH (X_3^2) were significant in showing smaller *p*-values of 0.02, 0.01, and 0.01, respectively. Thus temperature was the primary factor in the activity of Cytolase PCL 5 in the production of compound K rather than time or pH. The relationships between the response and the experimental levels of each factor were visualized by 3D response surface plots based on the equation derived (Fig. 1). It is obvious from the plot that the maximum yield of compound K (2.068 mg/mL) can be achieved by 78.05 h of the process at 55.36 °C at pH 4.30. Under this optimum condition, the experimental yield of compound K was 2.065 ± 0.175 mg/mL, in good accordance with the prediction.

In this study, we optimized the conditions for the practical enzymatic transformation method using commercially available pectinases. Cytolase PLC5 made possible the most effective production of compound K.

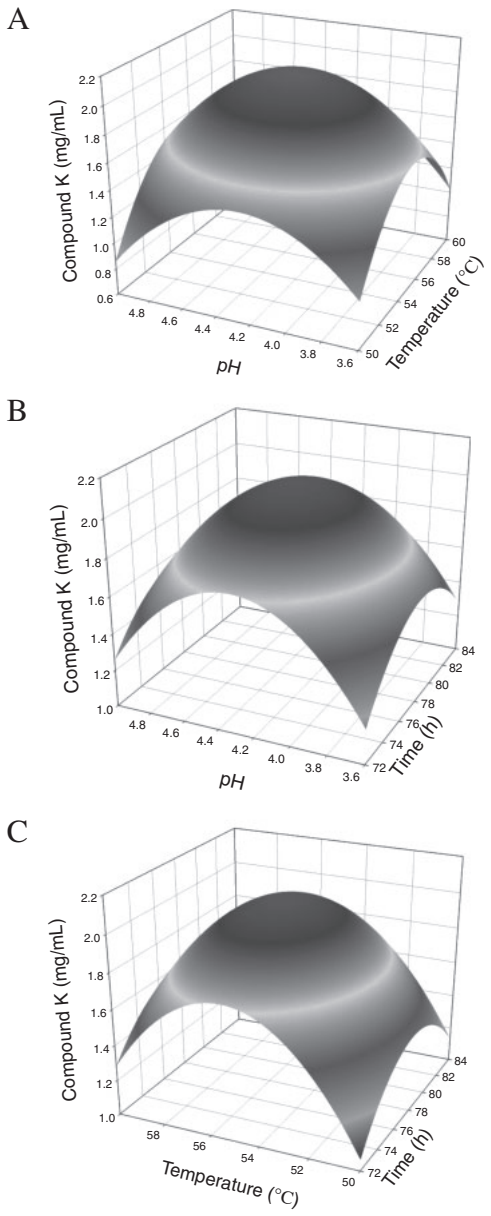

Fig. 1. Response Surface Plots Showing the Combined Effects of pH and Temperature (A), pH and Time (B), and Temperature and Time (C) on the Yield of Ginsenoside Compound K from Ginseng Extract.

Thus this finding suggests that the optimization of the single-step enzymatic process employing multiple enzymes is a practical method of accumulating the intermediate material of a complex hydrolysis pathway.

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