Supplementary material

Transformation of *Pueraria candollei* var. *mirifica* phytoestrogens using immobilized and free β-glucosidase, a technique for enhancing estrogenic activity

Fonthip Makkliang,[a] Wipawee Juengsanguanpornsuk,[b] Suppalak Phaisan,[c] Attapon Sakdamas,[c] Waraporn Putalun,[b] Seiichi Sakamoto,[d] Gorawit Yusakul,[c, e] *

[a] *School of Languages and General Education, Walailak University, Nakhon Si Thammarat, Thailand*

[b] *Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen, Thailand*

[c] *School of Pharmacy, Walailak University, Nakhon Si Thammarat, Thailand*

[d] *Graduate School of Pharmaceutical Sciences, Kyushu University, Higashi-ku, Fukuoka, Japan*

[e] *Biomass and Oil Palm Center of Excellence, Walailak University, Nakhon Si Thammarat, Thailand*

*Corresponding author: Gorawit Yusakul

School of Pharmacy, Walailak University, Nakhon Si Thammarat 80160, Thailand

Tel: +66(0)75-67-2839, Email address: gorawit.yu@mail.wu.ac.th, gorawit.yu@wu.ac.th

1.1 The analysis of β-glucosidase activity

The activity of β-glucosidase was monitored using *p*-nitrophenyl-β-D-glucopyranoside (*p*-NPG) as a substrate and *p*-nitrophenol (*p*-NP) was also used as a reference standard of enzymatic product. The β-glucosidase enzyme was diluted with 50 mM sodium citrate phosphate buffer pH 5. The solution of *p*-NPG (2.5 mM) was dissolved in ultrapure water. The *p*-NP solutions with serial concentration were prepared in the mixture (1:1) of 50 mM sodium citrate phosphate buffer pH 5 and 50 mM sodium carbonate buffer pH 9.6. The reaction mixtures included β-glucosidase (50 μL) solution and 2.5 mM *p*-NPG (50 μL), which were mixed and incubated in 96 well-plate (n=3) at room temperature for 10 min. Then, 50 mM sodium carbonate buffer pH 9.6 (100 μL) was added to stop the reaction. The absorbance was measured at 405 nm using a microplate spectrophotometer. The enzyme activity was calculated from the calibration curve of *p*-NP. A unit of enzyme is the amount releasing one μmole of *p*-NP per minute.

1.2 Analysis of surface morphology using a scanning electron microscope

The surface morphology of immobilized β-glucosidase alginate beads was observed using a scanning electron microscope (ZEISS Merlin® FE-SEM, Germany). The blank alginate bead and β-glucosidase alginate bead were allowed to be dried at 25 °C for 3 hours, and then water was removed by exchange with EtOH. A supercritical reactor of CO_2 was applied to dry alginate beads, then coated with gold using an auto coater (Cressington Scientific Instruments Ltd., Watford, UK). These gold-coated samples were subjected to a scanning electron microscope (SEM) (Carl Zeiss, Oberkochen, Germany) for morphological analysis at magnification power (10000×).

1.3 Instrumental and high-performance liquid chromatography for isoflavonoids analysis

The chromatographic analysis was performed using high-performance liquid chromatography coupled with ultra-violet detection (HPLC-UV) on a Thermo Scientific Dionex Ultimate 3000 (Thermo Scientific, MA, USA) with a variable wavelength detector (VWD-3100), an autosampler (WPS-3000SL), column compartment (TCC-3000SL) and a tertiary pump (LPG-4300SD). The analysis of target compounds employed a Vertical C_{18} column (5 μm particle size, 250 mm × 4.6 mm I.D., Vertical Chromatography Co., Ltd, Bangkok, Thailand). The conditions of the HPLC-UV system for the analysis of isoflavonoids are as follow; gradient elution with a constant flow rate of the mobile phase at 1.0 mL min^{-1} was applied to separate the isoflavonoids. The 1.0% acetic acid (A) and 60% acetonitrile (B) solutions were used as a mobile phase, which started 30% to 40% B with 0 to 7 min and increased from 40% to 70% B over 7 to 10 min, then kept at this constants ratio until 25 min. The solution B of the mobile phase was increased from 70% to 100% B with 25 to 28 min, after that, decreased to 30% B with 32 min and kept at this condition until 35 min for equilibration of the following analysis. The detection wavelength of the ultra-violet detector was set at 254 nm.

The HPLC-UV was performed to separate and determine five isoflavonoids. The chromatogram of puerarin (t_R=5.6 min), daidzin (t_R=7.9 min), genistin (t_R=11.8 min), daidzein (t_R=14.8 min), and genistein (t_R=18.8 min) were showed well peak separation with high resolution and good sharp peak. The linearities of puerarin, daidzin, genistin,

daidzein, and genistein were obtained of the concentration range from 1.56 to 50 μg mL^{-1}. The limit of detection (LOD) and limit of quantification (LOQ) were calculated using $3.3S_B/m$ and $10S_B/m$, respectively (Where S_B is the standard deviation of y-intercept, m is the slope of calibration curve). The LOD and LOQ were 0.047 and 0.141 μg mL^{-1} for puerarin, 0.122 and 0.368 μg mL^{-1} for daidzin, 0.227 and 0.687 μg mL^{-1} for genistin, 0.171 and 0.517 μg mL^{-1} for daidzein and 0.119 and 0.360 μg mL^{-1} for genistein.

Table S1 Central Composition Design (CCD) of RSM for the reaction between *Pueraria candollei* var. *mirifica* extract and β-glucosidases

Factor	Symbol	Unit	Level				
			-Alpha (-1.68)	**Low (-1)**	**Medium (0)**	**High (1)**	**+Alpha (+1.68)**
Independent variables							
pH	X_1	-	1.64	3	5	7	8.36
Temperature	X_2	°C	16.36	30	50	70	83.64
EtOH concentration	X_3	%	1.59	5	10	15	18.41
Dependent variables							
Immobilized β-glucosidase							
DZe	Y_1	μg mL^{-1}					
GTe	Y_2	μg mL^{-1}					
Free β-glucosidase							
DZe	Y_3	μg mL^{-1}					
GTe	Y_4	μg mL^{-1}					

Table S2 The design experiment matrix of central composition design (CCD) and their responses

Run	Independent variables			Responses			
	X_1	X_2	X_3	Y_1	Y_2	Y_3	Y_4
1	5.00	50.00	10.00	40.9±0.24	3.44±0.01	34.6±0.25	2.80±0.24
2	5.00	50.00	10.00	45.5±0.29	3.78±0.06	33.4±1.31	2.68±0.11
3	3.00	30.00	5.00	25.0±0.13	1.31±0.03	18.3±1.39	1.13±0.09
4	5.00	50.00	10.00	43.3±0.01	3.47±0.02	35.8±0.81	2.83±0.05
5	7.00	70.00	5.00	12.8±0.07	1.07±0.03	8.74±0.58	0.82±0.07
6	1.64	50.00	10.00	17.8±0.11	1.86±0.02	16.2±0.15	1.54±0.02
7	3.00	70.00	15.00	14.0±0.10	1.53±0.01	11.7±0.25	1.39±0.01
8	5.00	83.64	10.00	13.6±0.09	1.14±0.01	12.5±1.01	1.17±0.11
9	5.00	16.36	10.00	20.2±0.03	1.73±0.01	11.5±0.30	1.16±0.03
10	8.36	50.00	10.00	14.1±0.06	1.59±0.01	13.3±0.01	1.62±0.02
11	7.00	30.00	15.00	17.4±0.16	1.72±0.04	11.9±0.13	1.45±0.03
12	7.00	70.00	15.00	12.3±0.02	1.43±0.01	11.5±0.61	1.42±0.11
13	5.00	50.00	10.00	40.8±0.44	3.18±0.05	39.1±1.03	3.09±0.23
14	5.00	50.00	10.00	43.1±0.10	3.31±0.01	42.5±0.71	3.58±0.10
15	5.00	50.00	1.59	11.1±0.46	2.08±0.07	44.6±1.64	3.60±0.12
16	5.00	50.00	10.00	42.8±0.48	3.29±0.05	37.3±0.42	3.13±0.02
17	3.00	70.00	5.00	14.8±0.08	1.31±0.05	10.2±0.92	1.10±0.10
18	5.00	50.00	18.41	19.8±0.38	2.56±0.11	33.7±0.54	3.57±0.12
19	7.00	30.00	5.00	22.3±0.27	1.49±0.03	9.66±0.97	1.02±0.05
20	3.00	30.00	15.00	28.5±1.62	2.17±0.17	20.5±0.06	1.97±0.02

Supplement Figures

Fig. S1

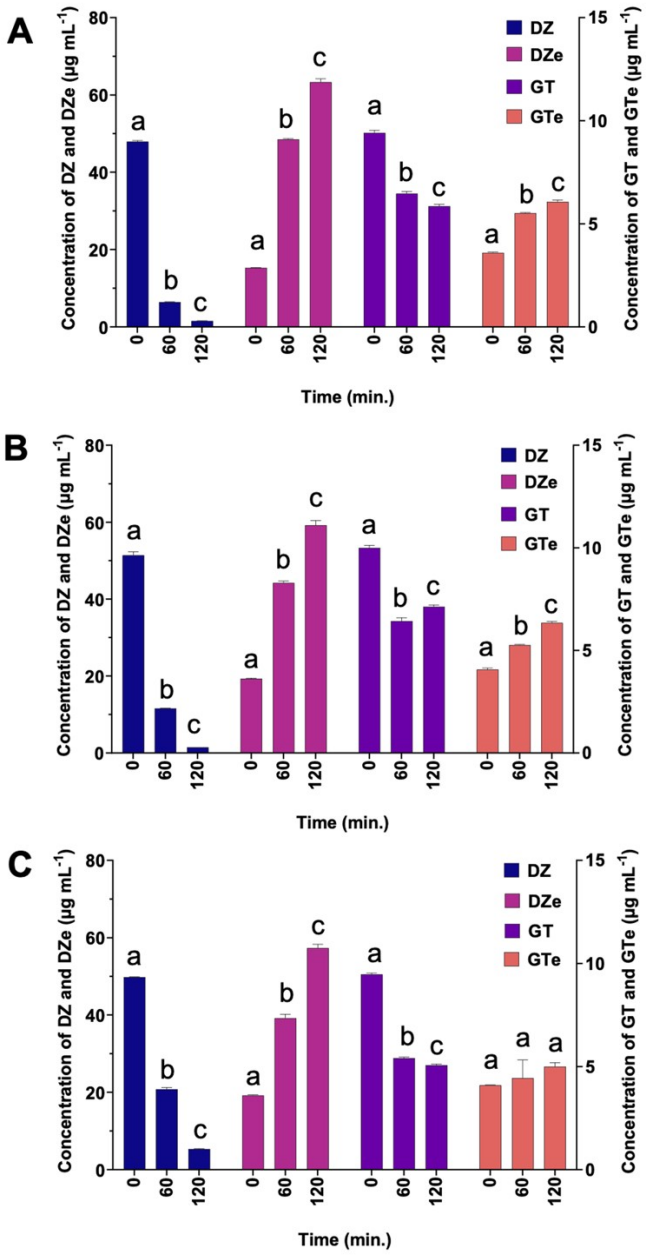

Fig. S1 Daidzin (DZ) and genistin (GT) are converted into daidzein (DZe) and genistein(GTe), respectively. The A, B, and C panels demonstrate the reactivity of β-glucosidase beads fixed in 2, 2.5, and 3.0% (w/v) alginate beads, respectively. The same and different letters (lowercase) indicate nonsignificant and significant changes when the data is compared between the reaction time of each compound. To determine statistical significance, one-way ANOVA was employed, followed by LSD (p<0.01).

Fig. S2

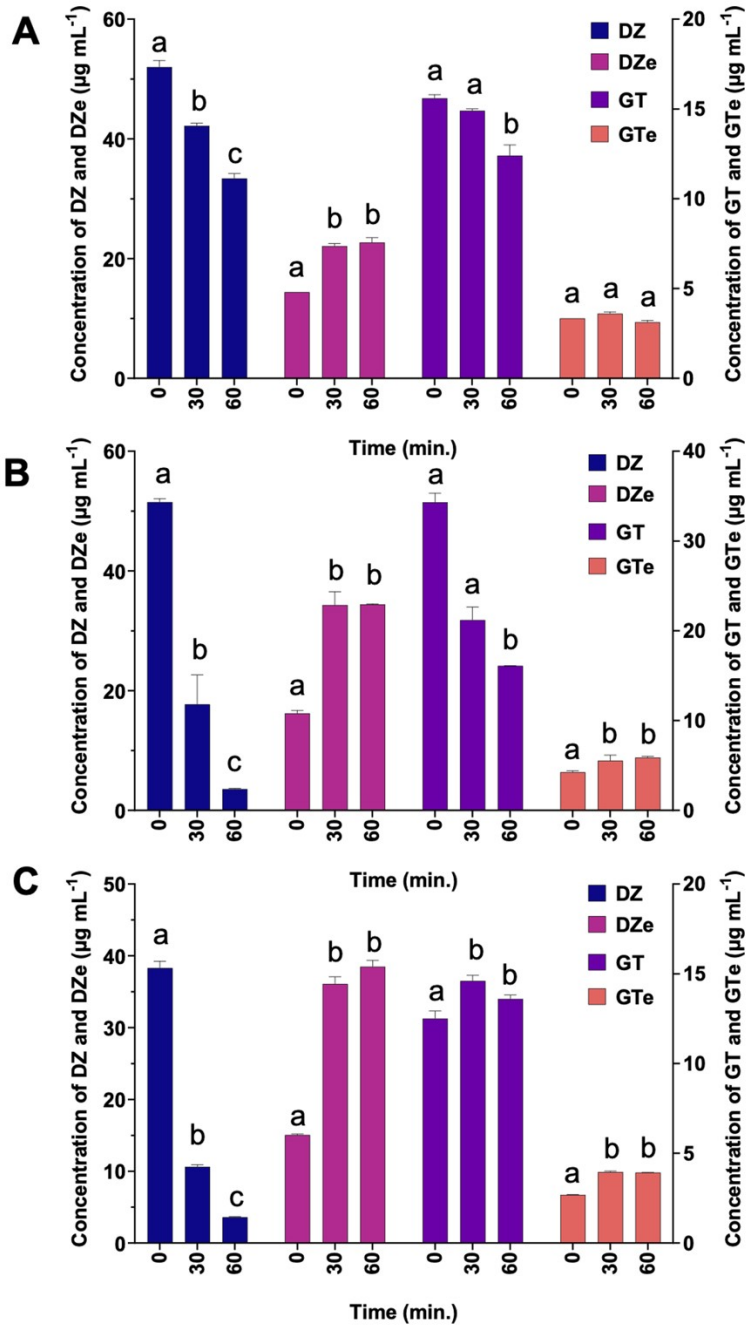

Fig. S2 Daidzin (DZ) and genistin (GT) are converted into daidzein (DZe) and genistein (GTe), respectively. The A, B, and C panels demonstrate the reactivity of β-glucosidase at 0.04, 0.2, and 1.0 U mL^{-1}, respectively. The same and different letters (lowercase) indicate nonsignificant and significant changes when the data is compared between the reaction time of each compound. To determine statistical significance, one-way ANOVA was employed, followed by LSD (p<0.01).

Fig. S3

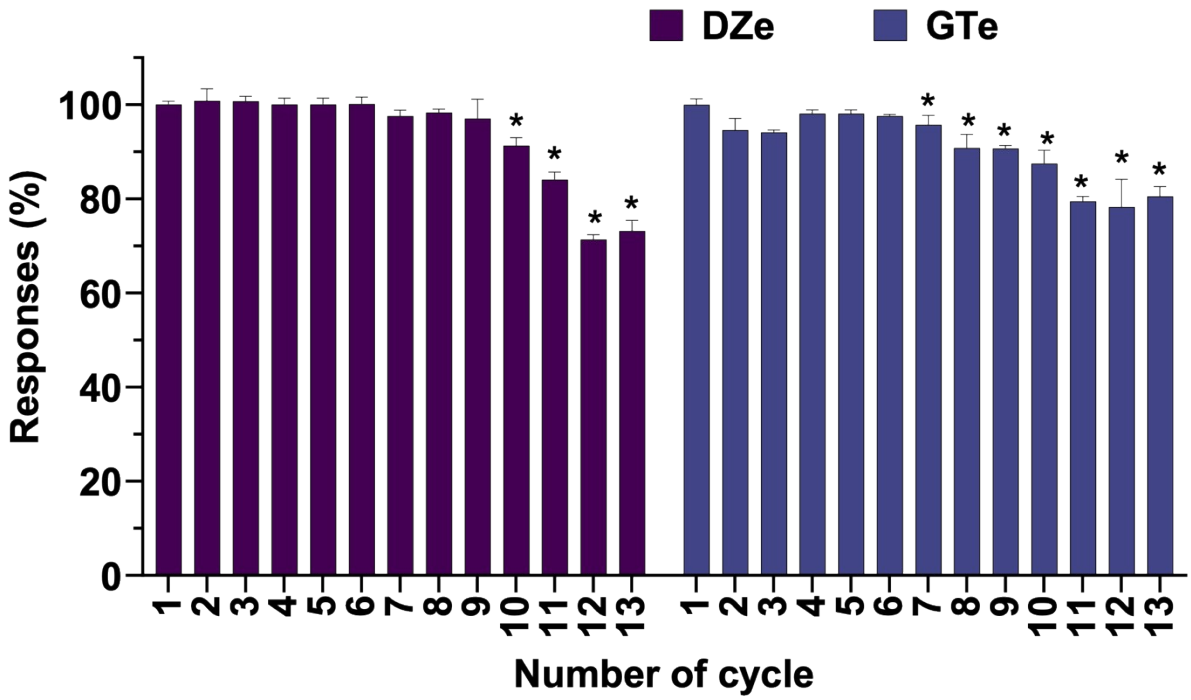

Fig. S3 Reusability of immobilized β-glucosidases in the production of daidzein (DZe) and genistein (GTe), where the first cycle of the reaction was set as 100% catalytic efficiency. The * denotes a significant difference in catalytic efficiency as compared to the first reaction cycle. One-way ANOVA was used to evaluate statistical significance, followed by LSD (p<0.01).