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ARTICLE

Optimizing Time and Temperature of Enzymatic Conversion of Isoflavone Glucosides to Aglycones in Soy Germ Flour

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Five factors (enzyme concentration, substrate concentration, pH, incubation temperature, and incubation time) were initially screened for the conversion of isoflavone glucosides to aglycones in soy germ flour. The incubation temperature/time most significantly affected aglycone yield; subsequently, a full 5 (35, 40, 45, 50, and 55 °C) × 6 (1, 2, 3, 4, 5, and 6 h) factorial design and response surface methodology were employed to attain an optimal incubation time/temperature condition. The optimum condition producing soy germ flour with a high concentration of daidzein, glycitein, and genistein was as follows: soy germ flour:deionized water (1:5, w/v), β-glucosidase at 1 unit/g of soy germ flour, pH 5, and incubation temperature/time of 45 °C/5 h. Under this optimal condition, most isoflavone glucosides were converted to aglycones with daidzein, glycitein, and genistein of ≥15.4, ≥6.16, and ≥4.147 μmol/g, respectively. In contrast, the control soy germ flour contained 13.82 μmol/g daidzin, 7.11 μmol/g glycitin, 4.40 μmol/g genistin, 1.56 μmol/g daidzein, 0.52 μmol/g glycitein, and 0.46 μmol/g genistein.

KEYWORDS: Soy germ flour; isoflavone aglycones; isoflavone glucosides; β-glucosidase; Plackett–Burman design; RSM

INTRODUCTION

Soybean and soy foods contain numerous phytochemicals. Soybean is a popular health food that has been consumed in Asian countries for many centuries (*1*). The current consumption of soybean and its soy products worldwide is due to their nutritional properties and the health-promoting characteristics of functional compounds such as isoflavones (*2*). The amount of isoflavones in soy germ or the hypocotyl is about 5−6 times higher than in the cotyledon (*3*, *4*). Therefore, soy germ can serve as a health-promoting ingredient for the food and supplement markets (*4*), because it is the most concentrated source of isoflavones in soybean.

Isoflavones are known for their biological activities including estrogenic, antifungal, and antioxidant activities (*5*). Isoflavones exist in the form of aglycones (daidzein, genistein, and glycitein) and β-glucoside conjugates, which include glucosides, malonylglucosides, and acetylglucosides. The main isoflavones in unprocessed soy flour are daidzin and genistin, which are the glucoside forms of daidzein and genistein (*6*, *7*). The aglycones are structurally similar to the mammalian estrogen oestradiol-17 β and, therefore, mimic the function of oestridiol in the human body (*8*). The β-glucosides have less estrogenic activity when compared to their respective aglycone forms (*9*, *10*). The aglycones were absorbed faster and in higher amounts than their glucosides by humans because of their hydrophilic capacity and lower molecular weight (*9*).

When soy foods are consumed, the glucosides are broken down by the bacteria in the intestine during the digestive processes to yield the aglycones (*11*). Isoflavones are not generally destroyed by heat but are rather subject to introconversion between different forms (*12*) and are stable at temperatures up to 260 °C (*4*). During soybean processing, daidzin, genistin, and glycitin can be converted to daidzein, genistein, and glycitein, respectively. When soybeans are soaked in water, the endogenous *β-glucosidase* enzyme hydrolyzes the isoflavone glucoside forms to their aglycone forms (*13*). Under acidic conditions, the glycones can be deconjugated to give aglycones. In addition, under acidic or basic conditions, the acetyl and malonyl groups can also be removed (*14*). The conversion of glucoside to aglycone form results in an increased amount of health-promoting compound in soy products. The consumption of isoflavones such as genistein and daidzein (aglycone forms) is potentially related with human health benefits, including reducing certain risk parameters of cardiovascular disease, relieving menopausal symptoms, contributing to bone growth or stabilization, and reducing the risk of several cancers in women such as breast and colon cancer (*15*, *16*).

Pandjaitan et al. (*7*) investigated conversion of genistin to genistein in defatted soy flour by added *β-glucosidase* enzyme (1−8 unit/g defatted soy flour) and reported 41−100% conversion yield. Our preliminary work demonstrated a conversion yield of 33−80% with the nondefatted soy germ flour. Thus, the

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Table 1. Plackett−Burman Design for Selection of Significant Factors Affecting Concentration of Isoflavone Aglycones

		factors and their levels[a]						
treatments	sampling	A	B	C	D	E	F	G
1	5	+4	+6	+50	−1	+20	−	−
2	4	+4	+6	−37	+4	−5	−	+
3	7	+4	−5	+50	−1	−5	+	+
4	1	−1	+6	−37	−1	+20	+	+
5	6	+4	−5	−37	+4	+20	+	−
6	8	−1	−5	+50	+4	+20	−	+
7	3	−1	+6	+50	+4	−5	+	−
8	2	−1	−5	−37	−1	−5	−	−

[a] (A) Concentration of enzyme (unit/g soy germ flour): high level, 4; low level, 1. (B) pH value: high level, 6; low level, 5. (C) Incubation temperature (°C): high level, 50; low level, 37. (D) Incubation time (h): high level, 4; low level, 1. (E) Concentration of substrate (g/100 mL): high level, 20; low level, 5. F and G are dummy variables: + indicates a high level; − indicates a low level.

objective of this study was to systematically optimize specific processing conditions (incubation temperature and time) to produce soy germ flour with a maximized isoflavone aglycone concentration using the Plackett−Burman design and response surface methodology (RSM).

MATERIALS AND METHODS

Materials. Germ from Thai soybean variety (cv. Chiang Mai 60) was obtained from Juuboonyong Agriculture Co., Ltd., Thailand. This variety was chosen because it is commercially available, and on the basis of our preliminary study, it contains a high level of total isoflavone content. The *β-glucosidase* enzyme (classification number: G0395) was purchased from Sigma Chemical Co. (St. Louis, MO). Isoflavone glucoside standards (genistin, glycitin, and daidzin) and isoflavone aglycone standards were purchased from LC Laboratories (Woburn, MA). Other reagents were analytical grade and purchased from Fisher Scientific (Pittsburgh, PA,) and Sigma Chemical Co.

Preparation of Soy Germ Flour. Soy germ flour was prepared by grinding the commercial soy germ (cv. Chiang Mai 60) using a pin mill (Alpine Mill, Augsburg, Germany). The soy germ flour was sieved through an 80 mesh sieve, collected, packed in plastic (HDPE) bags, and stored in cardboard boxes at 5 °C until further analysis. Two separate batches of soy germ flour were prepared.

Initial Screening of Significant Factors by the Plackett−Burman Design. The eight-run Plackett−Burman design was employed to screen/select the two most critical factors (enzyme concentration, substrate concentration, pH, incubation temperature, and/or incubation time) for conversion of isoflavone glucosides to aglycones as shown in **Table 1**.

Hydrolysis of Soy Germ Flour. Hydrolysis of soy germ flour was performed according to the method of Matsuura et al. and Pandjaitan et al. (*5*, *7*). The enzyme solution [1 unit (a low level) or 4 unit (a high level) of *β-glucosidase* enzyme in 0.5 mL] was added to dispersion of soy germ flour in deionized water (soy germ flour: deionized water in 1:5 or 1:20, w/v) according to the designed experiment in **Table 1**. The dispersion was adjusted to pH 5 or 6 due to the optimum pH range of the *β-glucosidase* enzyme toward the substrate (*5*, *7*) and incubated at the temperature and time following **Table 1**. Immediately after the required incubation time was reached, the dispersion was adjusted to pH 7.0 and centrifuged at 7000*g* for 15 min. The residue was collected, washed once with deionized water, and freeze-dried for further analysis.

High-Performance Liquid Chromatography (HPLC) Analysis of Isoflavones. The control and treated soy germ flours were separately extracted at 65 °C for 2 h in 80% aqueous methanol solvent and then saponified with NaOH at ambient temperature. Each mixture was then acidified by 1 mL of glacial acetic acid, filtered, diluted with water to 50% methanol, and centrifuged. The supernatant was collected and then injected into a reversed-phase HPLC system (*17*).

A HPLC solvent gradient system was used according to the method of Griffith and Collison (*18*). The HPLC system consisted of a Hewlett-Packard 1100 series HPLC (Agilent Technologies, Forest Hill, Vic., Australia) with an autosampler, a quaternary pump, a diode array ultraviolet (UV) visible detector, and Lichrospher 100RP-18 (250 mm × 4.6 mm internal diameter, 5 μm) reversed phase column. For the linear HPLC gradient, mobile phase A was combined water + methanol + 0.1% glacial acetic acid (88 + 10 + 2), and mobile phase B was combined methanol + 0.1% glacial acetic acid (98 + 2). An injection of a 10 μL of sample was followed by an increase of mobile phase B from 20 to 100% and a decrease of mobile phase A from 80 to 0% in 25 min. The solvent flow rate was 0.8 mL/min. The chromatograms obtained at a wavelength of 260 nm were used to quantify isoflavone concentration. All analyses were performed in duplicate.

Table 2. Effects of Enzyme Concentration, pH, Incubation Temperature and Time, and Substrate Concentration on Isoflavone Concentration from the Plackett−Burman Design

	μmol/g											
	daidzin		glycitin		genistin		daidzein		glycitein		genistein	
factors[a]	high	low	high	low	high	low	high	low	high	low	high	low
A	4.04	4.07	2.43	2.44	1.64	1.67	12.13	12.10	4.37	4.34	3.23	3.22
B	4.06	4.05	2.45	2.42	1.65	1.66	11.74	12.49	4.25	4.45	3.18	3.27
C	3.65	4.46	2.11[b]	2.76[b]	1.56	1.75	12.66[c]	11.57[c]	4.71[c]	4.00[c]	3.41[c]	3.04[c]
D	3.20[b]	4.91[b]	1.83[b]	3.03[b]	1.39[b]	1.92[b]	13.90[c]	10.33[c]	5.44[c]	3.26[c]	3.85[c]	2.60[c]
E	4.38	3.73	2.62	2.24	1.72	1.59	12.02	12.21	4.29	4.42	3.14	3.31

[a] (A) Concentration of enzyme (unit/g soy germ flour): high level, 4; low level, 1. (B) pH value: high level, 6; low level, 5. (C) Incubation temperature (°C): high level, 50; low level, 37. (D) Incubation time (h): high level, 4; low level, 1. (E) Concentration of substrate (g/100 mL): high level, 20; low level, 5. [b] Significant negative effect at $P < 0.20$ using a t test ($t_{\alpha\ 0.20\ \text{at df=2}} = 1.886$). [c] Significant positive effect at $P < 0.20$ using a t test ($t_{\alpha\ 0.20\ \text{at df=2}} = 1.886$).

Table 3. t Values Indicating a Significant Estimated Effect of Enzyme Concentration, pH, Incubation Temperature and Time, and Substrate Concentration on Isoflavone Concentrations from the Plackett−Burman Design Shown in **Table 2**

factors[a]	daidzin	glycitin	genistin	daidzein	glycitein	genistein
A	−0.077	−0.018	−0.207	0.046	0.148	0.148
B	0.005	0.106	−0.041	−1.382	−1.153	−0.705
C	−1.654	−2.208[b]	1.574	2.013[c]	4.167[c]	2.708[c]
D	−3.509[b]	−4.225[b]	−4.351[b]	6.606[c]	12.915[c]	9.238[c]
E	1.345	1.344	1.077	−0.362	−0.739	−1.261

[a] (A) Concentration of enzyme (unit/g soy germ flour): high level, 4; low level, 1. (B) pH value: high level, 6; low level, 5. (C) Incubation temperature (°C): high level, 50; low level, 37. (D) Incubation time (h): high level, 4; low level, 1. (E) Concentration of substrate (g/100 mL): high level, 20; low level, 5. [b] Significant negative effect at $P < 0.20$ using a t test ($t_{\alpha\ 0.20\ \text{at df=2}} = 1.886$). [c] Significant positive effect at $P < 0.20$ using a t test ($t_{\alpha\ 0.20\ \text{at df=2}} = 1.886$).

Statistical Analysis. The Plackett−Burman design was employed to screen/select critical factors. The main effects of each factor on isoflavone aglycone contents were estimated as the difference between averages of measurements made at the higher level and at the lower level. The significance of each factor was determined via a t test. Preliminary results (**Tables 2** and **3**) revealed that incubation temperature and time most significantly affected aglycone concentration. Therefore, these two factors were selected for optimization study.

For optimization of incubation time and temperature by RSM, a full 5 (35, 40, 45, 50, and 55 °C) × 6 (1, 2, 3, 4, 5, and 6 h) factorial design and RSM were employed to attain an optimal incubation time and temperature condition at a given pH (5.0), a level of substrate concentration (a dispersion of 1:5 w/v of soy germ flour:deionized water), and *β-glucosidase* level (1 unit/g of soy germ flour) for maximized isoflavone aglycone contents. A total of 30 experiment trials were conducted (**Table 4**). The data obtained from RSM on isoflavone aglycone contents were subject to analysis of variance (ANOVA). The experimental results of RSM were fitted via the response surface regression procedure, using the following second order polynomial equation:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_{11}X_1^2 + b_{22}X_2^2 + b_{12}X_1X_2$$

Table 4. Experimental and Predicted Responses for Isoflavone Glucoside and Aglycone Contents[a] in a 5 × 6 Full Factorial Design

			isoflavone glucosides (μmol/g)						isoflavone aglycones (μmol/g)					
			daidzin		glycitin		genistin		daidzein		glycitein		genistein	
Trt[b]	temp[b] (°C)	time[b] (h)	Obs.[c]	Pre.[c]	Obs.	Pre.	Obs.	Pre.	Obs.	Pre.	Obs.	Pre.	Obs.	Pre.
	control		13.82		7.11		4.40		1.56		0.52		0.46	
1	35	1	7.53	8.02	4.75	5.22	2.89	2.96	8.06	6.95	2.09	1.39	1.76	1.41
2	35	2	6.66	6.66	4.31	4.36	2.54	2.55	10.45	9.71	3.07	2.83	2.41	2.26
3	35	3	5.64	5.61	3.81	3.68	2.30	2.21	11.38	11.72	3.68	3.88	2.85	2.89
4	35	4	5.06	4.86	3.48	3.19	2.02	1.95	13.23	12.99	4.67	4.55	3.42	3.30
5	35	5	4.41	4.42	2.93	2.89	1.78	1.77	12.98	13.51	4.76	4.84	3.41	3.48
6	35	6	4.17	4.28	2.67	2.78	1.62	1.67	12.82	13.29	4.59	4.74	3.34	3.44
7	40	1	7.19	7.10	4.69	4.62	2.70	2.67	8.03	8.86	2.20	2.35	1.79	2.03
8	40	2	6.08	5.79	4.03	3.77	2.49	2.28	11.29	11.48	3.61	3.76	2.75	2.85
9	40	3	4.86	4.79	3.24	3.11	1.90	1.96	12.92	13.36	4.53	4.78	3.29	3.43
10	40	4	4.10	4.09	2.70	2.63	1.71	1.72	15.45	14.50	5.34	5.42	3.67	3.80
11	40	5	3.45	3.70	2.19	2.35	1.50	1.56	14.90	14.89	5.83	5.68	4.02	3.94
12	40	6	3.29	3.60	2.05	2.26	1.40	1.47	14.78	14.53	5.64	5.55	3.80	3.86
13	45	1	6.50	6.37	4.20	4.12	2.24	2.45	8.59	10.05	2.49	3.00	1.96	2.43
14	45	2	4.91	5.11	3.32	3.28	2.10	2.07	12.94	12.55	4.48	4.37	3.26	3.20
15	45	3	3.67	4.15	2.38	2.64	1.74	1.77	14.10	14.29	5.36	5.37	3.78	3.75
16	45	4	3.46	3.50	2.05	2.18	1.54	1.55	15.25	15.30	6.07	5.97	4.10	4.07
17	45	5	3.33	3.16	1.91	1.91	1.46	1.41	16.23	15.55	6.75	6.20	4.47	4.18
18	45	6	3.23	3.11	1.87	1.84	1.39	1.34	15.57	15.06	6.28	6.04	4.19	4.06
19	50	1	5.91	5.82	3.89	3.72	2.35	2.30	10.46	10.54	3.19	3.32	2.71	2.59
20	50	2	4.30	4.61	2.79	2.90	1.91	1.94	14.17	12.90	5.15	4.67	3.65	3.32
21	50	3	3.34	3.70	2.02	2.27	1.55	1.65	14.58	14.51	5.75	5.63	3.89	3.83
22	50	4	3.11	3.10	1.75	1.83	1.48	1.45	14.99	15.38	6.15	6.20	4.06	4.12
23	50	5	2.67	2.80	1.61	1.58	1.44	1.32	16.17	15.50	6.88	6.40	4.43	4.18
24	50	6	2.63	2.80	1.54	1.52	1.40	1.27	15.51	14.88	6.55	6.20	4.16	4.02
25	55	1	5.57	5.46	3.62	3.43	2.36	2.21	10.74	10.30	3.50	3.33	2.58	2.53
26	55	2	3.94	4.30	2.28	2.63	1.86	1.86	13.64	12.53	5.18	4.64	3.48	3.22
27	55	3	3.17	3.44	1.82	2.02	1.50	1.60	13.85	14.01	5.79	5.57	3.59	3.69
28	55	4	3.09	2.88	1.68	1.59	1.38	1.41	14.02	14.75	5.96	6.11	3.69	3.93
29	55	5	2.61	2.63	1.46	1.36	1.29	1.30	14.36	14.74	6.15	6.27	3.86	3.95
30	55	6	2.57	2.68	1.36	1.31	1.21	1.27	13.81	13.98	5.72	6.05	3.66	3.75

[a] At a given pH of 5 and *β-glucosidase* level of 1 unit/g soy germ flour. [b] Trt, treatment; temp, incubation temperature; time, incubation time. [c] Obs., observed value; Pre., predicted value.

where Y is the predicted response, b_0 is the value of the fitted response at the center point of the design [point (0,0)], b_1 and b_2 are linear regression terms, b_{11} and b_{22} are quadratic regression terms, and b_{12} is the cross-product regression term (*19*).

The statistical software package, SPSS (SPSS Inc., an IBM Co., Chicago, IL), was used for regression analysis of the experimental data, and STATISTICA (StatSoft, Inc., Tulsa, OK) was used to plot the response surface graphs. The quality of fit of the second-order polynomial model equation was expressed in the form of a contour plot, in order to illustrate the relationship between the responses and the experimental levels of each of the variables utilized in this study.

RESULTS AND DISCUSSION

Initial Screening of Significant Factors Using the Plackett−Burman Design. A total of five factors (enzyme concentration, substrate concentration, pH, incubation temperature, and/or incubation time) were screened for effective conversion of isoflavone glucosides to aglycones using the eight-run Plackett−Burman design. The concentrations (μmol/g) of isoflavone glucosides and their corresponding aglycones at various processing conditions are shown in **Table 2**. Factors were considered to have a significant effect on the response (i.e., isoflavone concentration) when the calculated t value was greater than 1.886 at $\alpha = 0.20$ (**Table 3**). According to **Tables 2** and **3**, the main effect of enzyme concentration (1−4 unit/g soy germ flour), pH, and substrate concentration was not significant. In contrast, incubation time (a low level at 1 h; a high level at 4 h) inserted a significant negative effect on all isoflavone glucosides (daidzin, glycitin, and genistin), but a significant positive effect on all isoflavone aglycones (daidzein, glycitein, and genistein) (**Tables 2** and **3**). Incubation temperature (a low level at 37 °C; a high level at 50 °C) inserted a significant negative effect only on glycitin, but a significant positive effect on all isoflavone aglycones (daidzein, glycitein, and genistein) (**Tables 2** and **3**). Therefore, the optimum level of these two factors (incubation temperature and time) was further determined by an RSM design.

Similar results reported by Xie et al. (*20*) were that the amounts of daidzein and genistein in soymeal produced under a condition with added 1.5, 2, 3, 5, or 7 units of *β-glucosidase* enzyme were not significantly different ($p < 0.05$). For the effect of pH on the conversion of glucosides to aglycones, Pandjaitan et al. (*7*) reported that the enzyme showed a more constant pH range of hydrolytic activity. There was no significant difference ($p < 0.05$) in the genistein concentration produced by exogenous β-glucosidase enzyme in soy protein concentrate at pH 4.0, 5.0, and/or 6.0.

Optimization of Incubation Temperature and Time by RSM. **Table 4** and **Figure 1** show the effects of incubation temperature and time on the concentration (μmol/g) of isoflavone glucosides and isoflavone aglycones in soy germ flour. Without being treated (control), soy germ flours contained the following concentrations of isoflavones: daidzin (13.82 μmol/g), glycitin (7.11 μmol/g), genistin (4.40 μmol/g), daidzein (1.56 μmol/g), glycitein (0.52 μmol/g), and genistein (0.46 μmol/g). The concentrations of the three glucosides (daidzin, glycitin, and genistin) decreased with increasing incubation time and temperature (**Figure 1a1**,**b1**, **c1**). In contrast, the three aglycones (daidzein, glycitein, and

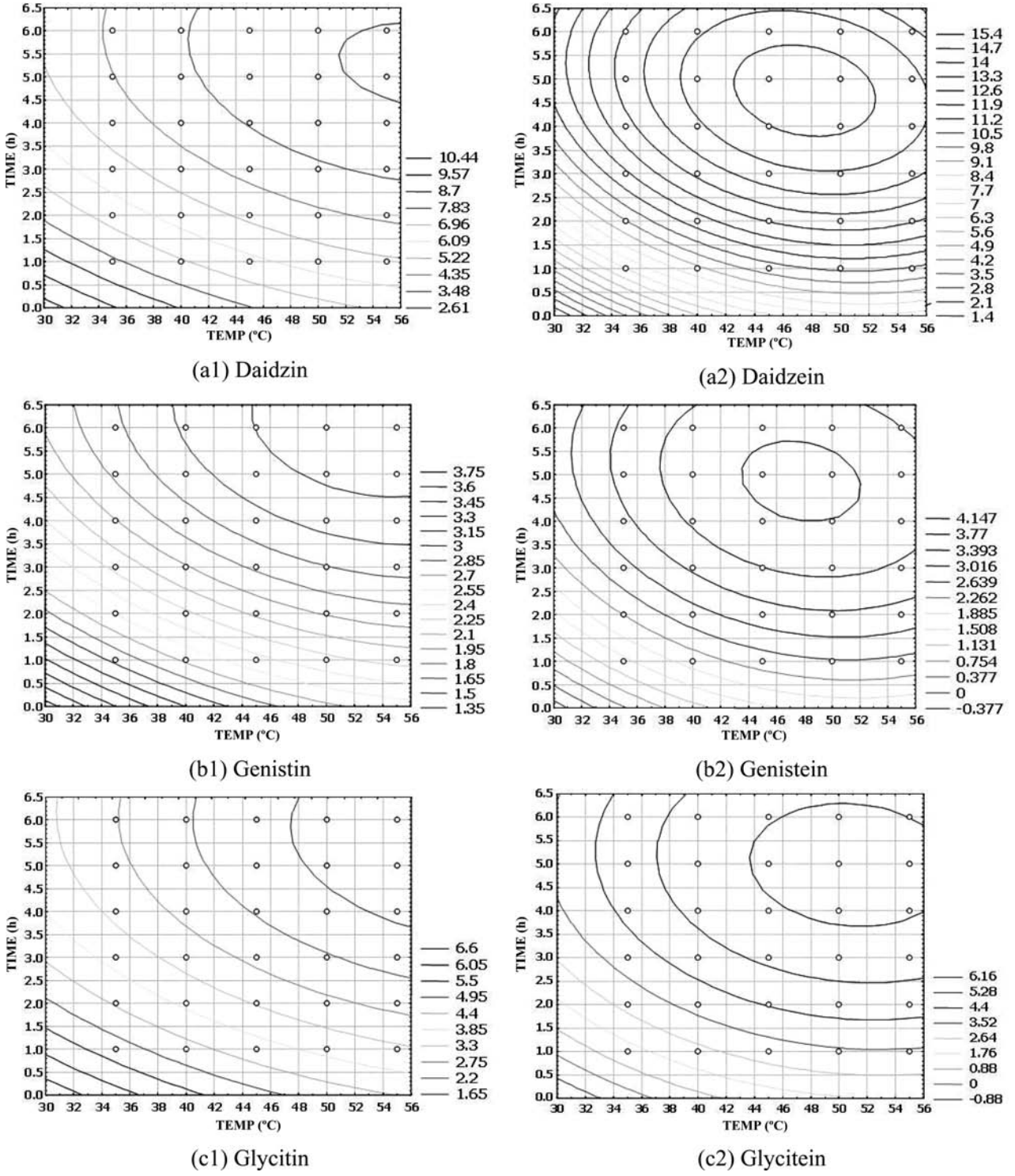

Figure 1. Contour plots comparing isoflavone glucoside and isoflavone aglycone contents (μmol/g) as affected by an interaction between incubation temperature and time at a given pH (5.0) and the β-*glucosidase* level (1 unit/g soy germ flour).

genistein) increased with increasing incubation time and temperature [**Figure 1a2**,**b2**,**c2**) as a result of conversion of their corresponding glucosides (**Figure 1a1**,**b1**,**c1**).

Several researchers also demonstrated that isoflavone glucosides can be converted to isoflavone aglycones depending on incubation temperature and time during soaking (*21*, *22*). Furthermore, they reported that the concentration of isoflavone aglycones generally increased with increased incubation temperature and time. An increased in isoflavone aglycone concentration was attributed to an increased amount of isoflavone glucosides, which was readily transformed to aglycones through hydrolysis by the β-*glucosidase* enzyme. The transformation of malonylglucosides and acetylglucosides to glucosides may provide an elucidation for the increased concentration of aglycones. Coward et al. (*23*) concluded that de-esterification of malonyl and acetyl conjugates can occur at high temperatures, which leads to the formation of glucosides. Malonylglucoside conjugates are thermally unstable and transformed to methyl malonate or methyl acetate and the isoflavone glucosides at high temperatures.

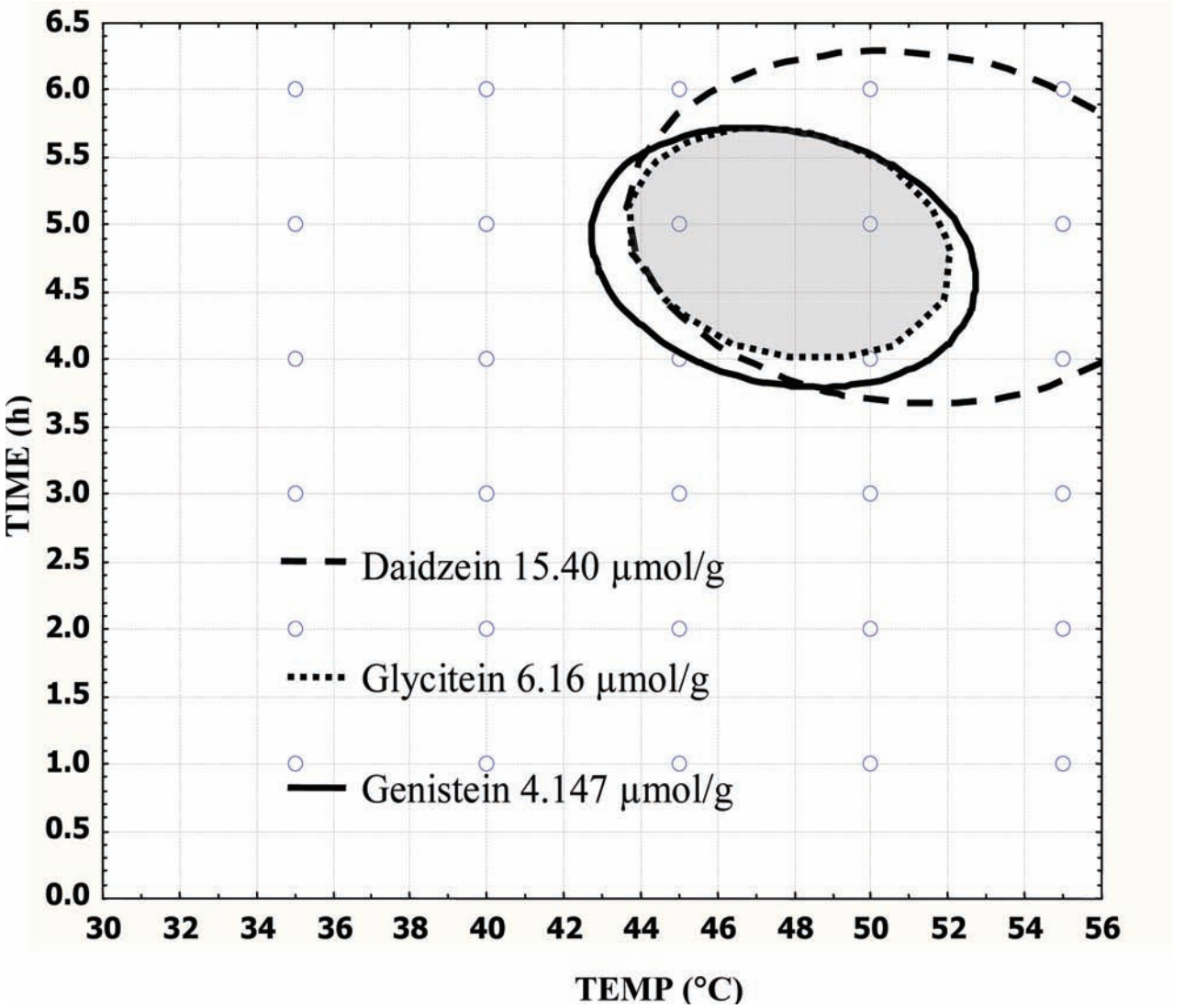

Figure 2. Shaded area indicating an optimum region of incubation time and temperature that would yield maximized daidzein, glycitein, and genistein concentrations at a given pH (5.0) and a *β-glucosidase* level (1 unit/g soy germ flour).

In this study, RSM was employed to attain an optimal incubation temperature and time condition that would yield maximal daidzein, glycitein, and genistein concentrations. The two independent variables were incubation temperature (X_1 = 35−55 °C) and incubation time (X_2 = 1−6 h). Dependent variables were daidzin (Y_1), daidzein (Y_2), glycitin (Y_3), glycitein (Y_4), genistin (Y_5), and genistein (Y_6). The high regression coefficient value (R^2) as shown below indicated that the variables were highly fitted to the regression equation for daidzin (R^2 = 0.976), daidzein (R^2 = 0.924), glycitin (R^2 = 0.971), glycitein (R^2 = 0.956), genistin (R^2 = 0.965), and genistein (R^2 = 0.939).

$$Y_1 = 21.6115 - 0.4705X_1 - 2.1467X_2 + 0.0037X_1^2 + 0.0096X_1X_2 + 0.1519X_2^2 \quad R^2 = 0.976$$

$$Y_2 = -30.886 + 1.4813X_1 + 4.809X_2 - 0.0143X_1^2 - 0.0266X_1X_2 - 0.3729X_2^2 \quad R^2 = 0.924$$

$$Y_3 = 13.5682 - 0.2817X_1 - 1.2644X_2 + 0.0021X_1^2 + 0.0032X_1X_2 + 0.0949X_2^2 \quad R^2 = 0.971$$

$$Y_4 = -16.3724 + 0.6793X_1 + 2.2366X_2 - 0.0064X_1^2 - 0.0063X_1X_2 - 0.1922X_2^2 \quad R^2 = 0.956$$

$$Y_5 = 7.3931 - 0.1581X_1 - 0.6539X_2 + 0.0013X_1^2 + 0.0035X_1X_2 + 0.0391X_2^2 \quad R^2 = 0.965$$

$$Y_6 = -10.7672 + 0.4781X_1 + 1.4715X_2 - 0.0046X_1^2 - 0.0081X_1X_2 - 0.1116X_2^2 \quad R^2 = 0.939$$

According to **Figure 2**, the optimal incubation time and temperature was around 44−52 °C and 4−5.5 h, respectively, with the predicted maximal concentration value of ≥15.4 μmol/g for daidzein (100% conversion of daidzin), ≥ 6.16 μmol/g for glycitein (79.32% conversion of glycitin), and ≥4.147 μmol/g for genistein (83.80% conversion of genistin). Using the above equations for prediction, for instance at 45 °C incubation temperature and 5 h incubation time, daidzein, glycitein, and genistein were predicted to be 15.55 μmol/g (observed value = 16.23 μmol/g), 6.20 μmol/g (observed value = 6.75 μmol/g), and 4.18 μmol/g (observed value = 4.47 μmol/g), respectively.

Validation of the Model and Implication. The validation of the statistical model and regression equations was conducted with a

Table 5. Comparison of Predicted Values against Observed Values on Isoflavone Aglycone Concentrations (μmol/g) within the Optimum Incubation Temperature and Time

incubation		daidzein (μmol/g)		glycitein (μmol/g)		genistein (μmol/g)	
temp (°C)	time (h)	Obs.[a]	Pre.[a]	Obs.	Pre.	Obs.	Pre.
45	5.5	16.27	15.90	6.99	7.04	4.48	3.36
46	5.0	15.78	16.14	6.91	7.16	4.41	3.37
47	4.1	15.68	16.05	6.59	7.07	4.42	3.28
47	5.4	15.98	16.04	6.75	7.24	4.46	3.32
47	5.7	15.50	15.86	6.74	7.18	4.57	3.28
48	4.0	15.40	16.04	6.36	7.11	4.44	3.23
48	5.0	15.67	16.20	6.83	7.33	4.66	3.31
50	5.4	15.75	15.98	6.89	7.43	4.73	3.17

[a] Obs., observed value; Pre., predicted value.

selected range of incubation temperatures (45−50 °C) and times (4.1−5.5 h) in the optimum regions (**Table 5**). Under these selected optimized conditions, the observed experimental value of daidzein, glycitein, and genitein was 15.40−16.27, 6.36−6.99, and 4.41−4.73 μmol/g, respectively (**Table 5**). The experimental values were similar to those predicted values, thus confirming the validity of the model.

In conclusion, the optimal and most practical condition to produce soy germ flour with maximized isoflavone aglycone concentrations was a fixed substrate concentration (a dispersion of 1:5 w/v of soy germ flour:deionized water), *β-glucosidase* concentration at 1 unit/g of soy germ flour, pH 5, and incubation temperature and time of 45 °C and 5 h. At this optimal condition, the daidzein, glycitein, and genistein to be obtained would be ≥15.4, ≥ 6.16, and ≥4.147 μmol/g, respectively. Certainly selecting a lower incubation temperature and time under this optimized condition would be more practical to economize the production cost of soy germ flour.

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