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Analytical Methods

Extraction yield of isoflavones from soybean cake as affected by solvent and supercritical carbon dioxide

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Abstract

Soybean cake has been shown to be a rich source of isoflavone and can be produced during processing of soybean oil as byproduct. The objectives of this study were to compare the extraction yield of isoflavone from soybean cake by solvent and supercritical carbon dioxide, and study the conversion of isoflavone glucosides to the biologically active aglycone by employing β-glucosidase. Results showed that with supercritical carbon dioxide extraction, a maximum yield of malonylglucoside and glucoside was generated at 60 °C and 350 bar, while a high level of acetylglucoside and aglycone was produced at 80 °C and 350 bar. Supercritical carbon dioxide extraction resulted in a lower yield of total isoflavone than solvent extraction, but the former was more applicable to extraction of acetylglucoside and aglycone, and the latter to malonylglucoside and glucoside. A peak level of aglycone was attained from conversion of isoflavone glucoside by β-glucosidase at 55 °C, pH 5, concentration 50 U/ml and incubation time of 2 h.

Keywords: Solvent extraction; Supercritical carbon dioxide; Isoflavone; Soybean cake; β-Glucosidase

1. Introduction

Isoflavones, a major class of flavonoids in soybean and soybean products, have been shown to be present in four chemical forms: aglycone (daidzein, genistein and glycitein), glucoside (daidzin, genistin and glycitin), acetylglucoside (acetyldaidzin, acetylgenistin and acetylglycitin), and malonylglucoside (malonyldaidzin, malonylgenistin and malonylglycitin). The beneficial effects of isoflavones in the prevention of chronic diseases such as coronary heart disease and osteoporosis, as well as alleviation of postmenopausal syndrome have been well established (Fritz, Seppanen, Kurzer, & Csallany, 2003; Lydeking-Olsen, Jensen, Setchell, Damhus, & Jensen, 2002; Setchell & Cassidy, 1999; Zubik & Meydani, 2003). However, most studies have indicated that the aglycones may possess higher biological activity than their corresponding glucose-containing isoflavones (Izumi et al., 2000; Setchell et al., 2003). Thus, it would be a great advantage to the health food industry if the various isoflavone glucosides in soybean can be converted to their aglycones.

Soybean cake is an important byproduct produced during soybean oil processing, and has been shown to be a rich source of isoflavone (Kao & Chen, 2006; Kao, Lu, & Chen, 2005). However, there is a paucity of information regarding how the isoflavones in soybean cake can be recovered. The extraction of isoflavones in food samples are often carried out by solvents. Several studies have shown that the extraction efficiency of isoflavones can be increased by using solvents like acetonitrile, methanol, ethanol and/or acetone in combination with 40–50% water or 10–20% acid (Kao & Chen, 2002; Rostagno, Palma, & Barroso, 2003). A high recovery can be achieved, but, organic solvents may pose safety and environmental pollution threats. Instead of solvent extraction, supercritical carbon dioxide extraction has become a popular technique in the functional food

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industry because of its non-toxic and environmentally friendly nature (Lee et al., 2005). But, the non-polar characteristic of carbon dioxide has limited its application to extraction of polar compounds. Nevertheless, the polarity of supercritical carbon dioxide can be enhanced by incorporating modifiers such as water and ethanol (Palma, Taylor, Zoecklein, & Douglas, 2000). Therefore, for extraction of polar isoflavones in soybean cake with supercritical carbon dioxide, it is necessary to add water or ethanol as modifier to increase extraction efficiency. In addition, the conversion of isoflavone glucosides to their corresponding aglycones in the presence of β-glucosidase under various conditions needs to be investigated. The objectives of this study were to: (1) compare the extraction efficiency of isoflavones in soybean cake by using solvent and supercritical carbon dioxide and (2) convert isoflavone glucosides in soybean cake to their aglycones, by employing β-glucosidase under various conditions.

2. Materials and methods

2.1. Materials

A total of 50 kg soybean cake was purchased from Chong-Liang Oil Co. (Taichung, Taiwan) and ground into powder and stored at −20 °C for use. Twelve isoflavone standards, including daidzein, genistein, glycitein, daidzin, genistin, glycitin, acetyldaidzin, acetylgenistin, acetylglycitin, malonyldaidzin, malonylgenistin and malonylglycitin were procured from LC Laboratories (Woburn, MA), Sigma (St. Louis, MO) and Nacalai (Kyoto, Japan). Internal standard formononetin was also from Sigma. The HPLC-grade solvent acetonitrile was from Merck (Darmstadt, Germany). Solvent ethanol (95%) was from Taiwan Tobacco and Wine Co. (Tainan, Taiwan). Deionised water was made, using a Milli-Q water purification system by Millipore Co. (Bedford, MA).

2.2. Instrumentation

The HPLC instrument was composed of two Jasco PU980 and PU1980 pumps (Jasco Co., Tokyo, Japan), a Jasco MD915 photodiode-array detector, a Rheodyne 7161 injector (Rheodyne LLC, Rohnert Park, CA) and an Agilent 1100 series G1316A column temperature controller (Palo Alto, CA). A Borwin software system was used to process data. A Vydac 201TP54 C18 column (250 × 4.6 mm I.D., particle size 5 μm; Grace, Deerfield, IL) was used to separate 12 isoflavones in soybean cake. A C18 guard column was from Phenomenex Co. (Torrance, CA). The supercritical carbon dioxide extractor (model Speed SFE 7010) was from Applied Separation Co. (Allentown, PA, USA), equipped with a 10-ml extraction vessel (40 × 14 mm I.D.) from Thermo Co. (Bellefonte, PA). The Sorvall RC5C high-speed centrifuge was from Du Pont Co. (Wilmington, DL).

2.3. Extraction of isoflavone from soybean cake by solvent

Fifty grams of soybean cake were poured into a 250 ml centrifuged bottle, followed by 150 ml of water:ethanol (1:1, v/v) and the mixture was shaken for 2 h. After centrifuging at 6000 rpm for 20 min (25 °C), the supernatant was collected and filtered through a glass fibre filter paper to obtain a total of 75 ml of filtrate. A portion (50 μl) of filtrate was collected and mixed with 100 μl of internal standard formononetin (200 μg/ml) and 850 μl of water:ethanol (1:1, v/v). The solution was mixed thoroughly and filtered through a 0.2 μm membrane filter, and a 20 μl sample was injected into the HPLC for isoflavone analysis.

2.4. Extraction of isoflavones from soybean cake by supercritical carbon dioxide

In this study we intended to compare the effects of different temperatures and pressures on the extraction efficiency of isoflavones from soybean cake. The experimental conditions were modified, based on a method by Rostagno, Araujo, and Sandi (2002). Initially, 1 g of soybean cake powder sample was poured into a 10 ml extraction vessel with 0.2 g of polypropylene cotton plugged on both sides, followed by 1.6 ml of ethanol:water (70:30, v/v) as modifier. A total of 12 treatments, including four temperatures (50, 60, 70 and 80 °C), in combination with three pressures (300, 350 and 400 bar) were selected for extraction. After the desired temperature and pressure was reached, a static extraction was carried out for 10 min, followed by dynamic extraction for 20 min at a flow rate of 1 ml/min. Next, 1.6 ml of ethanol:water (70:30, v/v) as modifier was injected into the vessel with a syringe, and static extraction was continued for 10 min and dynamic extraction for 20 min. Triplicate extractions were done for each sample and the volume of modifier used was 4.8 ml. After extraction, the vessel was flushed with 5 ml of ethanol:water (1:1, v/v) and diluted to 20 ml with the same solvent. A portion of extract (200 μl) was mixed with 100 μl of internal standard formononetin (200 μg/ml) and 700 μl of ethanol:water (1:1, v/v), after which the solution was filtered through a 0.2 μm membrane filter and 20 μl was injected into the HPLC for isoflavone analysis.

2.5. Conversion of isoflavone glucosides to aglycones

β-Glucosidase was used to measure the conversion efficiency of isoflavone glucosides to their corresponding aglycones under various conditions. In the beginning, the β-glucosidase activity was determined using a method, as described by Matsuura and Obata (1993). The substrate *p*-nitrophenol-β-D-glucoside (*p*-NPG) was selected for evaluation of β-glucosidase activity, as it can be converted to *p*-nitrophenol and quantified based on the absorbance at 420 nm. Seven concentrations of 20, 40, 60, 80, 100, 150 and 200 μM of *p*-nitrophenol in 0.1 M disodium hydrogen phosphate–citric acid buffer solution (pH 5) were prepared, and a portion of each *p*-nitrophenol solution (0.4 ml) was

collected and mixed with 0.1 ml of the same buffer solution, after which the mixture was reacted at 30 °C for 5 min, followed by adding 0.5 ml of 0.5 M aqueous sodium carbonate solution (4 °C), to form a deep yellow colour and the absorbance was measured at 420 nm. The standard curve of *p*-nitrophenol was prepared by plotting concentration against absorbance.

2.6. Assessment of optimum temperature of β-glucosidase

A concentration of 1 mM *p*-NPG was prepared in 0.1 M disodium hydrogen phosphate–citric acid buffer solution (pH 5). Likewise, 0.05 U/ml β-glucosidase was prepared in the same buffer solution. A 0.4 ml fraction of *p*-NPG solution was collected and incubated in a water bath for 10 min at 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75 and 80 °C, followed by adding 0.1 ml of β-glucosidase (0.05 U/ml) to each solution and reacting for a further 5 min. Then 0.5 ml of 0.5 M sodium carbonate solution (4 °C) was added to each solution to terminate the reaction and the absorbance was measured at 420 nm.

The absorbance was converted to concentration using the *p*-nitrophenol standard curve, with the β-glucosidase activity (unit) being calculated by dividing concentration (μM) of *p*-nitrophenol by reaction time (min). The optimum temperature was obtained from the curve by plotting the unit of β-glucosidase activity against temperature.

2.7. Assessment of optimum pH of β-glucosidase

A concentration of 1 mM *p*-NPG was prepared in disodium hydrogen phosphate–citric acid buffer with pH at 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5 and 8. Then 0.4 ml of each *p*-NPG solution was preheated in a 55 °C water bath for 10 min, followed by adding 0.1 ml of β-glucosidase (0.05 U/ml) and reacting at the same temperature for 5 min. Next, a 0.5 ml aliquot of 0.5 M sodium carbonate solution (4 °C) was added to terminate the reaction, and the absorbance was measured at 420 nm. Similarly, the absorbance was converted to concentration using the *p*-nitrophenol standard curve, with the β-glucosidase activity (unit) being calculated by dividing concentration (μM) of *p*-nitrophenol by reaction time (min). The optimum pH was obtained from the curve by plotting the unit of β-glucosidase activity against reaction time.

2.8. Assessment of conversion of isoflavone glucoside to aglycone

A 50 ml isoflavone extract from soybean cake was evaporated to dryness and dissolved in 20 ml of 0.1 M disodium hydrogen phosphate–citric acid buffer (pH 5). Six concentrations of 5, 10, 25, 50, 100 and 200 U/ml β-glucosidase were each prepared in the same buffer solution at 4 °C. Then 200 μl of isoflavone extract was mixed with 20 μl each of β-glucosidase and each mixture was reacted in a 55 °C water bath for 0.5, 1, 2, 3, 4, 5, 6, 7 and 8 h, after which 180 μl ethanol (4 °C) was added to terminate the reaction. A portion (50 μl) of each mixture was collected and mixed with 100 μl of internal standard formononetin (200 μg/ml), followed by adding 850 μl of ethanol:water (1:1, v/v). After filtering through a 0.2 μm membrane filter, a 20 μl sample was injected into the HPLC.

2.9. Analysis of isoflavone

The various isoflavones were identified by comparing retention times of unknown peaks with reference standards and co-chromatography with added standards. In addition, the absorption spectrum of each peak was scanned using a photodiode-array detector and compared with that of each standard.

For quantification, two concentrations of 200 and 2000 μg/ml for all 12 isoflavone standards and a level of 200 μg/ml internal standard formononetin were each prepared in methanol. Three levels of 1, 5 and 10 μg/ml of each isoflavone standard was prepared from the former (200 μg/ml) by mixing 25, 125 and 250 μl, respectively, with 500 μl of formononetin and diluting to 5 ml with methanol. Likewise, another three levels of 20, 50 and 100 μg/ml of each isoflavone standard were prepared from the latter (2000 μg/ml) by mixing 50, 125 and 250 μl, respectively, with 500 μl of formononetin and diluting to 5 ml with methanol. All the isoflavone standard solutions were filtered through a 0.2 μm membrane filter and a 20 μl sample was injected separately into the HPLC, using a method described by Kao and Chen (2006). The standard curves of 12 isoflavones were obtained by plotting concentration ratio (isoflavone standard/internal standard) against peak area ratio (isoflavone standard/internal standard), and the regression equation and correlation coefficient (r^2) were calculated. The amount of each isoflavone was then obtained using a formula as described in a previous study (Kao & Chen, 2002, 2006).

3. Results and discussion

3.1. Solvent extraction

After quantitation, soybean cake was found to contain malonylglucoside, glucoside, acetylglucoside and aglycone at 2411, 2184, 256 and 159 μg/g, respectively, a total of 5010 μg/g. The total amount of isoflavone was shown to be higher than some other reports by Wang and Murphy (1996), Wang, Ma, Pagadala, Sherrard, and Kishnan (1998), Coward, Smith, Kirk, and Barnes (1998), and Kao and Chen (2002), which may be caused by differences in variety, growth environment, harvest time, processing condition and storage condition of soybean.

3.2. Supercritical carbon dioxide extraction

3.2.1. Pressure effect

It has been well established that supercritical carbon dioxide solubility, a major factor responsible for extraction

efficiency, can be changed by changing the density, viscosity and diffusion characteristics of the supercritical fluid (Brogle, 1982). Table 1 shows the effect of various temperatures (50, 60, 70 and 80 °C) and pressures (300, 350 and 400 bar) on the extraction efficiency of isoflavones form soybean cake with ethanol:water (70:30, v/v) as modifier. At 50 °C, all 12 isoflavones showed the highest yield at 300 bar with the exception of malonylglycitin, which equalled to 589, 1697, 231 and 2828 μg/g for malonylglucoside, glucoside, acetylglucoside and aglycone, respectively, with a slight difference between 300 and 350 bar. However, a pronounced decline of all isoflavones occurred at 400 bar. Theoretically, the higher the pressure, the larger density and solubility the supercritical fluid, and better the extraction efficiency (Araujo, Silva, & Chaves, 2007). However, a high pressure may also decrease supercritical fluid diffusivity, leading to a lower extraction yield because of decreased interaction between supercritical fluid and sample (Macias-Sanchez et al., 2005). Thus, the diffusivity decrease should play a more important role in lowering yield of isoflavone at 400 bar than density increase. A similar outcome was reported by Macias-Sanchez et al. (2005), who found a pressure of 500 bar to be lower in extraction yield of chlorophyll A in microalga than at 400 bar at the same temperature. Nevertheless, the low extraction efficiency caused by isoflavone degradation at an elevated pressure cannot be ignored.

A different trend occurred at 60 °C, with a large yield of 798 and 1982 μg/g at 350 bar for malonylglucoside and glucoside, respectively, but a high content of acetylglucoside (342 μg/g) at 300 bar, and no significant difference in aglycone level between 300 bar (178 μg/g) and 350 bar (182 μg/g). The total amount of isoflavones followed the order: 350 bar (3279 μg/g) > 300 bar (2371 μg/g) > 400 bar (1949 μg/g). The same tendency also applied at 70 and 80 °C at 350 bar (Table 1). As explained above, a pressure of 300 bar may result in a lower density and solubility of supercritical carbon dioxide than at 350 bar, whereas a high pressure at 400 bar may cause a low diffusivity. This would account for a high yield of total isoflavone at 350 bar between 60 °C and 80 °C. In a similar study, Rostagno et al. (2002) reported that at 40, 50, 60 or 70 °C, a higher extraction yield of total isoflavone was obtained from soybean at 360 bar than at 300 bar. Likewise, a better extraction efficiency of daidzein and genistein from soybean hypocotyls was observed at 380 bar than at 261 bar (Araujo et al., 2007).

3.2.2. Temperature effect

The temperature effect on extraction efficiency of isoflavones should be discussed in combination with pressure effect. It has been well documented that at low pressure, the higher the temperature, the lower the supercritical fluid density and solubility, and extraction efficiency as well (Brunner, 2005; Palmer & Ting, 1995). Conversely, following a rise in temperature, a minor decline in supercritical fluid density was shown at an elevated pressure, which in turn resulted in better extraction efficiency, probably because of an enhancement of the solute vapour pressure effect (Brunner, 2005; Palmer & Ting, 1995). The effect of temperature on the yield of isoflavone in soybean cake is also shown in Table 1. With pressure at 300 bar, a temperature of 50 °C produced the largest yield of malonylglucoside, glucoside and aglycone, equal to 589, 1697 and 231 μg/g, respectively, but a lower level of acetylglucoside was found at 50 °C (311 μg/g) than at 60 °C (342 μg/g). Interestingly, the total isoflavone followed the order: 50 °C > 60 °C > 80 °C > 70 °C. This result implied that at low pressure, the higher the temperature was, the lower the extraction efficiency was. In addition to solubility, the temperature effect on isoflavone conversion or degradation should also be taken into consideration (Chien, Hsieh, Kao, & Chen, 2005), which may account for a significant loss of total isoflavone at 70 °C. However, compared to the other three temperatures, a higher content of malonylglucoside and glucoside at 80 °C indicated no isoflavone conversion occurred. Thus, the solubility effect of supercritical carbon dioxide at 300 bar and 80 °C should be more important than isoflavone conversion effect in terms of extraction yield of isoflavone.

In another study dealing with dry heating of soybean powder at 80 °C and 150 °C for 4 h, Murphy, Barua, and Hauck (2002) reported that at 80 °C a minor change of isoflavone glucoside occurred, while at 150 °C there was a fast conversion of malonylgenistin to acetylgenistin and genistin. Similarly, Chien et al. (2005) reported a large production of acetylgenistin and genistin from malonylgenistin standard during dry or wet heating at 150 °C and 200 °C. However, during heating of soy milk at 80 °C for 3 h, most malonylgenistin was converted to genistin (Murphy et al., 2002), demonstrating that isoflavone glucosides by wet heating are more susceptible to conversion or degradation than by dry heating. Hence, the temperature effect of supercritical carbon dioxide extraction on conversion or degradation of isoflavone glucoside in our study should be similar to dry heating.

Compared to a pressure of 300 bar, a different phenomenon was shown at 350 bar, that is, a large level of malonylglucoside (798 μg/g) and glucoside (1982 μg/g) was generated at 60 °C, whereas a high content of acetylglucoside (396 μg/g) and aglycone (274 μg/g) were produced at 80 °C. This outcome indicated that at elevated temperature (80 °C) and pressure (350 bar), malonylglucoside and glucoside could be converted to acetylglucoside and aglycone, respectively. In contrast, a low temperature (50 °C) treatment would reduce formation of both acetylglucoside and aglycone. Comparatively, the yields of high-polar malonylglucoside and glucoside were favoured at low temperature, whereas less-polar acetylglucoside and aglycone dominated at high temperature. Surprisingly, the total isoflavone followed a different tendency: 60 °C > 70 °C > 80 °C > 50 °C. In addition to isoflavone conversion, the isoflavone degradation at an elevated temperature may account for this effect. A similar phenomenon was also shown at 400 bar.

Table 1
Isoflavone contents (μg/g)[a] in soybean cake as extracted by supercritical carbon dioxide

Isoflavone	Treatment											
	50 °C			60 °C			70 °C			80 °C		
	300 bar	350 bar	400 bar	300 bar	350 bar	400 bar	300 bar	350 bar	400 bar	300 bar	350 bar	400 bar
Mdin	247 ± 3.2a	219 ± 5.6b	135 ± 10.2c	184 ± 7.8b	317 ± 2.3a	188 ± 10.1b	105 ± 7.6b	169 ± 4.8a	84.3 ± 3.5c	181 ± 17.9a	199 ± 14.7a	159 ± 2.8b
Mglin	151 ± 6.4b	173 ± 1.1a	70.0 ± 17.8c	86.0 ± 3.3b	241 ± 12.7a	83.9 ± 7.5b	52.5 ± 11.6b	137 ± 8.0a	47.8 ± 1.4b	87.9 ± 9.6a	81.3 ± 2.2a	51.0 ± 2.1b
Mgin	191 ± 4.9a	131 ± 3.9b	53.9 ± 15.9c	66.9 ± 9.9c	240 ± 4.9a	81.0 ± 1.1b	53.6 ± 9.4b	134 ± 5.4a	38.9 ± 1.3c	105 ± 11.9b	124 ± 5.4a	68.2 ± 14.5c
Sub total	589 ± 4.4a	523 ± 5.0b	259 ± 43.5c	336 ± 15.5b	798 ± 19.6a	353 ± 3.8b	211 ± 28.3b	440 ± 16.9a	171 ± 5.3c	374 ± 6.4b	404 ± 17.0a	278 ± 16.8c
Din	337 ± 13.7a	300 ± 3.6b	156 ± 5.1c	313 ± 5.9b	401 ± 26.7a	214 ± 6.6c	168 ± 3.8b	356 ± 8.3a	109 ± 3.8c	215 ± 1.9c	301 ± 9.2a	227 ± 1.5b
Glin	337 ± 18.9a	275 ± 9.9b	179 ± 4.9c	241 ± 16.2b	356 ± 9.4a	255 ± 14.1b	177 ± 4.2b	443 ± 20.7a	122 ± 3.7c	189 ± 14.3b	280 ± 5.4a	189 ± 7.1b
Gin	1023 ± 11.7a	970 ± 36.1b	510 ± 7.9c	960 ± 8.6b	1224 ± 24.4a	713 ± 23.8c	538 ± 18.0b	1090 ± 3.8a	374 ± 19.4c	695 ± 10.6b	954 ± 25.8a	697 ± 6.7b
Sub total	1697 ± 26.0a	1544 ± 25.4b	846 ± 12.7c	1514 ± 29.5b	1982 ± 44.4a	1182 ± 42.4c	882 ± 20.6b	1888 ± 14.2a	605 ± 25.9c	1099 ± 22.7b	1536 ± 28.0a	1112 ± 8.9b
Adin	85.9 ± 0.9a	77.5 ± 2.8b	56.8 ± 1.9c	93.0 ± 8.6a	87.4 ± 1.4a	69.2 ± 3.4b	63.5 ± 1.8b	83.5 ± 2.8a	49.1 ± 0.3c	64.7 ± 1.4b	85.7 ± 1.9a	65.9 ± 3.9b
Aglin	105 ± 1.4a	90.7 ± 5.3b	73.4 ± 2.6c	93.3 ± 2.2a	94.3 ± 6.2a	94.5 ± 4.2a	91.2 ± 9.5a	101 ± 6.4a	75.6 ± 2.6b	84.6 ± 7.0b	171 ± 14.6a	87.8 ± 5.9b
Agin	120 ± 2.2a	115 ± 5.6a	68.2 ± 4.0b	156 ± 6.5a	136 ± 4.3b	94.6 ± 8.4c	78.3 ± 3.9b	130 ± 4.6a	54.3 ± 2.4c	84.5 ± 1.3b	139 ± 2.3a	90.5± 8.6b
Sub total	311 ± 3.0a	283 ± 13.2b	198 ± 6.3c	342 ± 16.9a	317 ± 3.4b	258 ± 15.2c	233 ± 10.0b	314 ± 13.4a	179 ± 3.4c	234 ± 5.9b	396 ± 15.7a	244 ± 6.2b
Dein	85.3 ± 0.5a	65.4 ± 12.5b	34.1 ± 3.4c	58.8 ± 1.7b	67.3 ± 2.6a	51.9± 3.2c	41.2 ± 3.6b	77.0 ± 1.1a	25.8 ± 1.0c	40.9 ± 5.7b	82.3 ± 3.0a	42.6 ± 0.9b
Glein	84.9 ± 2.3a	56.6 ± 3.8b	49.5 ± 3.9c	64.4 ± 1.7a	56.3 ± 0.9b	49.2± 3.8c	53.8 ± 3.9b	70.2 ± 7.1a	43.3 ± 3.5c	46.8 ± 1.8c	109 ± 3.2a	58.9 ± 2.0b
Gein	60.8 ± 0.5a	50.1 ± 6.6b	28.6 ± 1.3c	55.1 ± 2.6a	58.1 ± 2.6a	54.6± 3.6a	30.0 ± 3.6b	59.4 ± 0.8a	27.9 ± 2.5b	47.2 ± 8.4c	82.8 ± 1.5a	56.2 ± 1.2b
Sub total	231 ± 2.2a	172 ± 22.4b	112 ± 3.6c	178 ± 2.5a	182 ± 4.2a	156± 8.4b	125 ± 7.4b	207 ± 8.6a	97.0 ± 2.3c	135 ± 15.0c	274 ± 6.6a	158 ± 1.9b
Total	2828 ± 32.5a	2522 ± 63.4b	1415 ± 48.1c	2371 ± 27.8b	3279 ± 66.0a	1949 ± 60.1c	1451 ± 28.2b	2849 ± 52.1a	1052 ± 31.3c	1841 ± 12.9b	2610 ± 46.6a	1792 ± 32.0c

Symbols bearing different letters (a–c) in the same row within each temperature are significantly different ($p < 0.05$).
Mdin: malonyldaidzin; Mglin: malonylglycitin; Mgin: malonylgenistin; Din: daidzin; Glin: glycitin; Gin: genistin; Adin: acetyldaidzin; Aglin: acetylglycitin: Agin: acetylgenistin; Dein: daidzein; Glein: glycitein; Gein: genistein.
[a] Mean of duplicate analyses ± standard deviation.

Collectively, temperature and pressure can affect the extraction yield by supercritical carbon dioxide, and an interaction ($p < 0.05$) existed between both as well. Although the supercritical carbon dioxide extraction temperature was lower than that of ordinary cooking or roasting, the isoflavone conversion or degradation can still occur when in combination with pressure. All in all, the amount of malonylglucoside declined following a rise in extraction temperature, probably because of a solubility decrease or conversion to acetylglucoside, glucoside or aglycone. The largest amount of total isoflavone (3279 μg/g) at 350 bar and 60 °C was much higher than in reports by Rostagno et al. (2002), and Araujo et al. (2007), in which only several isoflavones (daidzin, genistin, daidzein and genistein) were investigated. The difference in raw material (soybean or hypocotyl) and extraction conditions (360 bar and 50 °C, 380 bar and 60 °C) may account for this effect.

3.3. Comparison of solvent and supercritical carbon dioxide extraction

In comparison, solvent extraction resulted in a higher yield of malonylglucoside and glucoside than supercritical carbon dioxide extraction. But for acetylglucoside and aglycone, a higher level was obtained by supercritical carbon dioxide at 350 bar and 80 °C than by solvent extraction. Nonetheless, solvent extraction showed a much larger yield of total isoflavone than supercritical carbon dioxide extraction. Apparently the non-polar nature of supercritical carbon dioxide should be more applicable to extraction of both acetylglucoside and aglycone.

3.4. Evaluation of the optimum condition of β-glucosidase

Fig. 1 shows the effect of temperature and pH on activity of β-glucosidase from almond. Following a rise in temperature and pH, the β-glucosidase activity increased sharply and reached a plateau at 55 °C and pH 5, but a decline occurred afterwards. Compared to temperature, pH should exert a more pronounced effect on β-glucosidase activity, as indicated by the sharp pH curve. The optimum condition of β-glucosidase activity can be affected by source or purification method. Matsuura, Sasaki, and Murao (1995) isolated β-glucosidase C from soybean cotyledon and its

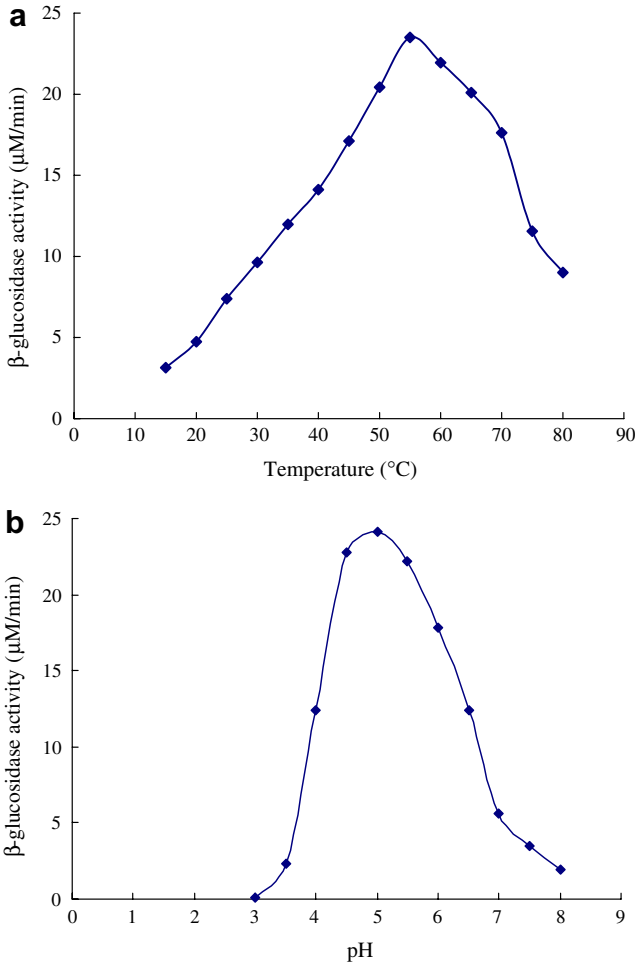

Fig. 1. Effects of temperature (a) and pH (b) on the activity of β-glucosidase from almond.

Table 2
Contents (μg/ml)[a] of aglycone isoflavone in soybean cake extract after incubating with β-glucosidase for varied length of time

Incubation time (h)	β-glucosidase (U/ml)[b]					
	5	10	25	50	100	200
0	142 ± 0fA	142 ± 0fA	142 ± 0fA	142 ± 0eA	142 ± 0eA	142 ± 0dA
0.5	620 ± 19eE	779 ± 24eD	910 ± 16eC	941 ± 9dAB	970 ± 29dA	908 ± 25cBC
1	712 ± 79dE	876 ± 25dD	936 ± 20dC	971 ± 7cB	994 ± 3cA	980 ± 32bABC
2	870 ± 28dD	953 ± 5cC	997 ± 13bcB	1032 ± 10bA	1046 ± 9bA	1039 ± 43abAB
3	882 ± 12dD	959 ± 11bcC	985 ± 11cB	1054 ± 36abA	1052 ± 24abA	1069 ± 13aA
4	907 ± 2cE	979 ± 15bD	991 ± 3cC	1065 ± 10aAB	1062 ± 2aB	1083 ± 13aA
5	965 ± 47bC	1003 ± 35abC	1014 ± 11abC	1066 ± 21aA	1072 ± 19abA	1084 ± 18aA
6	998 ± 19bD	1003 ± 26abCD	1034 ± 16aBC	1070 ± 26aAB	1072 ± 12aA	1084 ± 22aA
7	1002 ± 3bC	1012 ± 33abBC	1027 ± 26abBC	1076 ± 37abAB	1082 ± 9aA	1091 ± 13aA
8	1050 ± 1aB	1043 ± 20aB	1044 ± 10abB	1088 ± 17aA	1082 ± 19aA	1092 ± 32aA

[a] Average of duplicate analyses ± standard deviation.
[b] Symbols bearing different letters (a–f) in the same column are significantly different ($p < 0.05$). Symbols bearing different letters (A–E) in the same row are significantly different ($p < 0.05$).

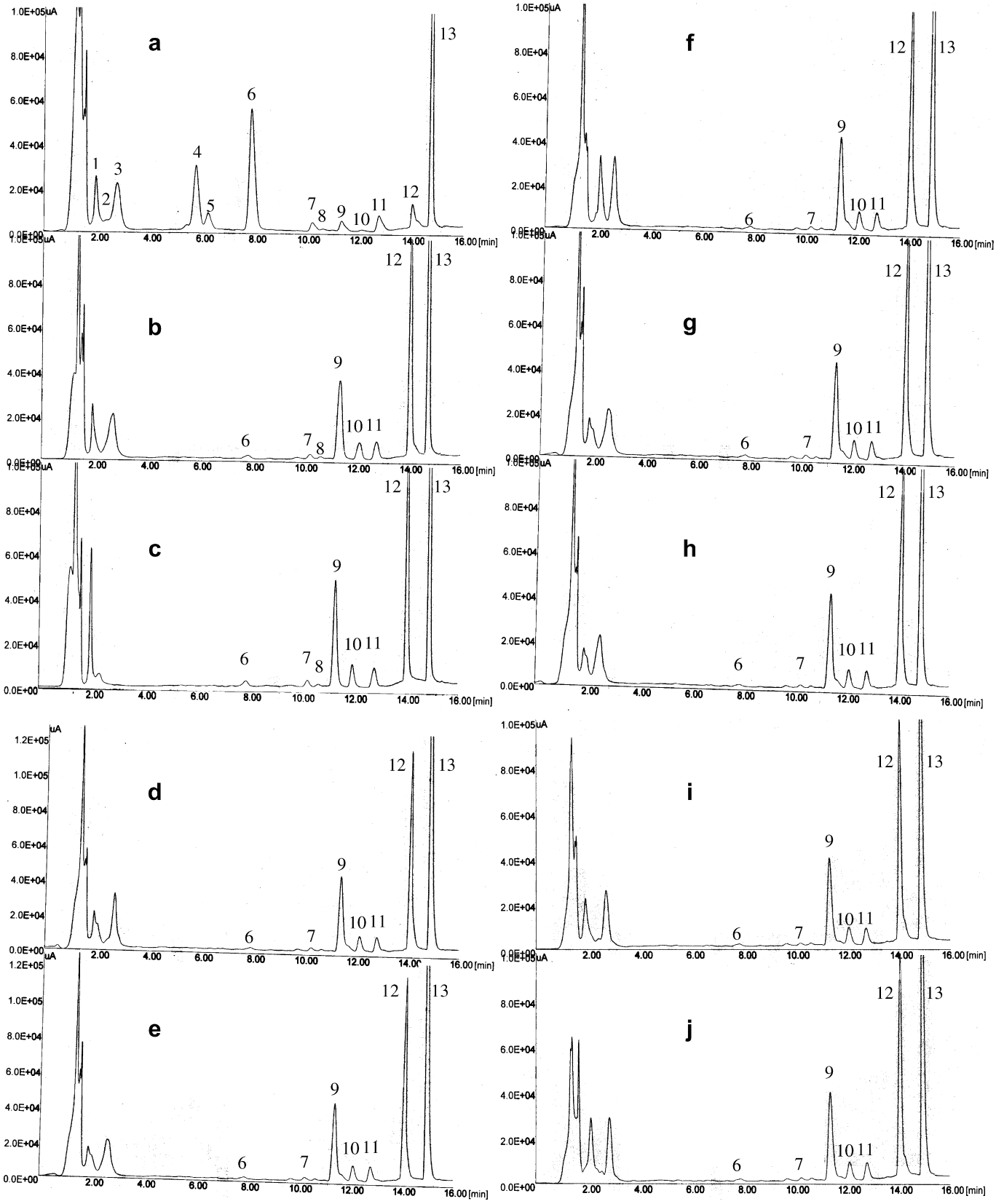

Fig. 2. HPLC chromatograms of soybean cake extract after incubating with 50 U/ml of β-glucosidase for 0–8 h. (a) 0 h; (b) 0.5 h; (c) 1 h; (d) 2 h; (e) 3 h; (f) 4 h; (g) 5 h; (h) 6 h; (i) 7 h; (j) 8 h. Chromatographic conditions described in text. Peaks: 1. malonyldaidzin, 2. malonylglycitin, 3. malonylgenistin, 4. daidzin, 5. glycitin, 6. genistin, 7. acetyldaidzin, 8. acetylglycitin, 9. daidzein, 10. glycitein, 11. acetylgenistin, 12. genistein, 13. formononetin (IS).

maximum activity was determined to be at 45 °C and pH 4.5. In a study dealing with conversion of genistin in soy protein concentrate to genistein by a commercial β-glucosidase, the most suitable conditions were found to be 50 °C and pH 5.0 (Pandjaitan et al., 2000). Similarly, the optimum condition for β-glucosidase produced from microorganism *L. delbrueckii* KCTC1047 was shown to be 45 °C and pH 6 (Choi, Kim, & Rhee, 2002).

The content of aglycone in soybean cake extract after incubating with different β-glucosidase concentrations for varying lengths of time is shown in Table 2 and Fig. 2. Prior to β-glucosidase treatment, both malonylglucoside and glucoside were the most abundant isoflavones in soybean cake extract, with acetylglucoside and aglycone constituting a small portion. With β-glucosidase concentration at 5 U/ml, the content of isoflavone glucoside decreased with increasing incubation time, accompanied by a sharp rise of aglycone from 142 to 1050 μg/ml over a period of 0–8 h. A similar trend was followed for the β-glucosidase concentration from 10 to 200 U/ml. However, for 25 U/ml β-glucosidase, almost all the isoflavone glucosides were hydrolysed in 0.5 h and thus a large amount of aglycone was formed (Table 2). Following an increase in β-glucosidase concentration from 50–200 U/ml, a fast conversion of isoflavone glucosides proceeded continuously and a peak was reached for aglycone in 2 h and remained almost the same even after prolonged incubation for 8 h (Table 2 and Fig. 2). Therefore, the most appropriate concentration and incubation time to achieve the maximum activity of β-glucosidase was chosen to be 50 U/ml and 2 h, respectively.

4. Conclusion

With supercritical carbon dioxide extraction, a large yield of malonylglucoside and glucoside was produced at 60 °C and 350 bar, while a high amount of acetylglucoside and aglycone was formed at 80 °C and 350 bar. Solvent extraction resulted in a greater yield of malonylglucoside and glucoside than supercritical carbon dioxide extraction, but for acetylglucoside and aglycone, a higher content was obtained for supercritical carbon dioxide extraction. The optimum condition for conversion of isoflavone glucoside to aglycone by β-glucosidase was 55 °C, pH 5, concentration 50 U/ml and incubation time 2 h.

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