# Enrichment of Two Isoflavone Aglycones in Black Soymilk by Immobilized $\beta$-Glucosidase on Solid Carriers

Kuan-I Chen,[†] Yi-Chen Lo,[†] Nan-Wei Su,[‡] Cheng-Chun Chou,[†] and Kuan-Chen Cheng*[,†,§]

[†]Graduate Institute of Food Science & Technology, National Taiwan University, No. 1, Sec. 4, Roosevelt Road, Taipei, Taiwan
[‡]Department of Agricultural Chemistry, National Taiwan University, Taipei 10617, Taiwan
[§]Institute of Biotechnology, National Taiwan University, No. 1, Sec. 4, Roosevelt Road, Taipei, Taiwan

**ABSTRACT:** A catalytic system for deglycosylation of isoflavone in black soybean milk was established. $\beta$-Glucosidase which was covalently immobilized onto the glass microspheres exhibited a significant efficiency for the conversion of pNPG to p-nitrophenol over other carriers. The optimum temperature for pNPG hydrolysis was 40 °C, and complete reaction can be reached in 30 min. Operational reusability was confirmed for more than 40 batch reactions. Moreover, the storage stability verification demonstrated that the glass microsphere catalytic system was capable of sustaining its highest catalytic activity for 40 days. The kinetic parameters, including rate constant ($K$) at which isoflavone glycosides deglycosylation were determined, the time ($\tau_{50}$) in which 50% of isoflavone glycosides deglycosylation was reached, and the time ($\tau_{complete}$) required to achieve complete isoflavone glycosides deglycosylation, were $0.35 \pm 0.04$ min$^{-1}$, $2.04 \pm 0.25$ min, and 30 min (for daidzin) and $0.65 \pm 0.03$ min$^{-1}$, $1.19 \pm 0.08$ min, and 20 min (for genistin), respectively. HPLC results revealed that this enzyme system took only 30 min to reach complete isoflavone deglycosylation and the aglycone content in the total isoflavones in black soymilk was enriched by $51.42 \pm 0.17\%$ under a 30 min treatment by the glass microsphere enzymatic system.

**KEYWORDS:** isoflavone deglycosylation, $\beta$-glucosidase, black soymilk, glass microspheres, immobilized enzyme system

## INTRODUCTION

Isoflavone, a well-known phytoestrogen, is a unique subgroup of flavonoids found most abundantly in cotyledon and hypocotyls of soybeans and soy derived foods.[1] The studies of isoflavone have recently drawn tremendous attention due to its potential health-enhancing benefits,[2] including reduction in cardiovascular disease,[3] cancer prevention,[4] osteoporosis prevention,[5] and high in antioxidant activities.[6] The amount of isoflavone has been reported to be ~1−5 mg/g in dry-soybean and ~10 mg/100 g in soy milk.[7] Studies have also revealed that isoflavones in their aglycone forms exhibit higher biological activity[8,9] and are more metabolically active that can be absorbed faster in greater amounts than their glycosides.[10,11] These results suggested that the intake of isoflavone aglycone-rich soy foods might be more effective for the purpose of healthy enhancement. For this reason, the enrichment of isoflavone aglycones in soy foods before consumption attracts growing attention.[12]

Soymilk is one of the traditional food beverages consumed popularly in Asian countries over the past decades. In recent years, soymilk has been consumed as a milk substitute by premenopausal women, milk allergy and lactose intolerance patients, and vegans.[13,14] Many studies have been carried out to enhance the total isoflavone aglycone content in soymilk fermented with probiotic microorganisms by means of endogenous $\beta$-glucosidase activity that can hydrolyze glucoside moieties.[12,15] The function of $\beta$-glucosidase is to remove the glucoside conjugates resulting in the accumulation of isoflavone aglycones. Because of the advantage of stabilizing microorganisms firmly on solid surfaces, immobilization of microorganisms becomes a common approach during soybean fermentation.[16−18] Despite of the fact that fermentation is beneficial for isoflavone bioconversion, some limitations still exist, such as the fact that the process is time-consuming and the difficulty in scaling up in food industry.

Recently, immobilized enzyme systems have been established in food industry to obtain specific products by catalyzing their corresponding substrates. Immobilized biocatalyst has several advantages over free enzyme system, including easy separation from reaction solution, reusability for reducing costs, continuous processing, and long-term stability.[19] The most famous example in food industry is the production of fructose syrup from glucose converted by immobilized glucose isomerase.[20,21] Diano et al. also reported a bed reactor packed with polyacrylonitrile (PAN) beads coated with pectin enzymes for the clarification of apple juice.[22] Moreover, some microscale[23] or nanoscale[24] materials have also been applied in the research of enzyme immobilization.

In the present study, we evaluated the feasibility of soybean milk isoflavone conversion using an immobilized $\beta$-glucosidase enzyme system. In this respect, $\beta$-glucosidase was immobilized on four different carriers (glass microspheres, nylon pellets, cellulose beads, and PAN beads) for deglycosylation of isoflavone in a more direct and faster manner, and 4-nitrophenyl $\beta$-D-glucuronide (pNPG) was used as an indicator. The carrier which possessed the most efficient catalytic behavior among these four solid carriers was chosen and applied to deglycosylation of isoflavone in black soymilk. The kinetic parameters of the best catalytic system for isoflavone deglycosylation were further determined as a function of different operative conditions. The efficiency of this immobilized

enzyme system was evaluated based on the changes of isoflavone content (genistin, daidzin, genistein, and daidzein) in black soymilk before and after deglycosylation.

## ■ MATERIALS AND METHODS

**Materials.** β-Glucosidase from *Aspergillus niger* was purchased from Sigma-Aldrich (St. Louis, MO). 4-Nitrophenyl β-D-glucuronide (*p*NPG) purchased from Alfa Aesar (Ward Hill, MA) was used as the substrate to determine the catalytic activity of immobilized enzyme system. Black soymilk was prepared by milling the black soybeans [*Glycine max* (L.) Merr.] (Tainan, No. 3) with a grinder where black soybeans were purchased from ShiaYing Farmers' Associations (ShiaYing, Tainan) and stored in a dried and cooled condition (used in three months). Resultant slurry was then filtered through a double-layered cheesecloth. Soymilk was autoclaved at 121 °C for 20 min.

Glass microspheres, nylon pellets, cellulose beads, and PAN beads were separately utilized as solid carriers for enzyme immobilization. Glass microspheres were purchased from Sigma-Aldrich with 9−13 μm particle size. Nylon 6/6 pellets were provided by Keen Crop Company (Tainan, Taiwan). Porous cellulose beads and porous PAN beads were synthesized as previously described.[22,25]

The utilization of nylon pellets,[22] cellulose beads,[26] and PAN beads[22] as enzyme immobilization carriers has been published previously. Glass spheres in microscale were new to the area of enzyme immobilization. The reduction of size particle in nylon pellets, cellulose beads, and PAN beads into the similar size as glass microspheres was limited. Glass microspheres with the microscale volume provide larger surface area for catalytic reaction. Owing to the different size of different carrier types, the only way is to fix the volume of 40 cm$^3$ for deglycosylation of isoflavone in 50 mL of black soymilk. It took longer reaction time when using lower volume of 20 cm$^3$ solid carriers (data not shown), and as a result, the volume of 40 cm$^3$ was the most effective volume to achieve complete deglycosylation.

**Carrier Preparation and Activation.** *Glass Microspheres.* A total volume of 40 cm$^3$ glass microspheres was first treated with 10% nitric acid (HNO$_3$) at 90 °C for 1 h. After several washes with distilled water, microspheres were subsequently immersed in a 10% 3-amino-propyltriethoxysilane (APES) aqueous solution (50 mL, pH 3.4) at 70 °C for another 3 h. Aminopropyl-glass was cleaned with distilled water and dried overnight in an oven at 80 °C.

*Nylon Pellets.* The procedure for nylon pellets modification has been described previously.[22] Briefly, a total volume of 40 cm$^3$ nylon pellets was immersed in 30 mL of dimethyl sulfate reagent for 4 min at 100 °C. A cleaning step was followed immediately by cold methanol (MeOH) washing and O-alkylated nylon pellets were then immersed in a 10% hexamethylenediamine (HMDA) aqueous solution (30 mL) at room temperature for 90 min.

*Cellulose Beads.* Cellulose acetate (6 g) was dissolved in an organic solution (acetone/DMSO = 6:4). The homogenized solution was added by a syringe and precipitated in water. The beads were then washed with distilled water and dried overnight at room temperature to a constant weight. A pretreatment of cellulose beads was carried out in 0.2 M NaOH for 90 min to enhance the hydroxyl groups on surface. After a rinse with distilled water, a total volume of 40 cm$^3$ of cellulose beads was activated with the treatment of 2.5% glutaraldehyde aqueous solution at 100 °C for 30 min.[26]

*PAN Beads.* PAN powder (18 g), LiNO$_3$ (1 g), and glycerin (3 g) were dissolved in 156 mL of dimethylformamide (DMF). The homogenized mixture was dropped by a syringe and precipitated in water. The beads were washed with distilled water and immersed in a 30% glycerin solution for 24 h. The beads were then dried at 70 °C in an oven to a constant weight. A total volume of 40 cm$^3$ of PAN beads was activated by treating 15% NaOH solution at 50 °C for 1 h. After thorough washing with distilled water, the beads were immersed in a 10% 1,2-diaminoethane solution (30 mL) for another 60 min at room temperature. The beads were then thoroughly washed once more with distilled water.

**Enzyme Immobilization.** Each activated carrier was treated with 2.5% glutaraldehyde aqueous solution at room temperature for 60 min.

The carriers were washed at room temperature with double-distilled water, and incubated separately for 16 h at 4 °C with 50 mg of β-glucosidase dissolved in 50 mL of 0.1 M phosphate buffer at pH 6. The unbound enzymes were removed from the carriers by washing with 0.1 M phosphate buffer, and enzymes linked carriers were stored at 4 °C.

The amount of enzymes being successfully immobilized on the carriers was determined by monitoring the difference between the amount of β-glucosidase initially used and the amount recovered in the solution at the end of the immobilization process. The concentration of β-glucosidase was determined according to Bradford dye-binding procedure.[27]

Five milligrams of *p*NPG, which is equal to the amount of isoflavone in 50 mL of soymilk, was dissolved in 0.1 M phosphate buffer (50 mL, pH 6). Each type of enzyme immobilized beads was incubated in 50 mL of *p*NPG solution in a batch reactor at 30 and 50 °C, respectively. A volume of 900 μL of *p*NPG solution was withdrawn, following with an addition of 1 mL of 2 M sodium carbonate (Na$_2$CO$_3$) to stop the reaction. The absorbance of *p*NPG was measured at 425 nm and recorded at regular time intervals. The release of *p*-nitrophenol converted from *p*NPG catalyzed by enzyme immobilized beads was used as an indicator to select the best catalytic enzyme system. The higher value of absorbance reflects the more efficiency in catalytic activity.

The procedures mentioned above including chemical modification and activation of each type of carriers, determination of the amount of unbound enzyme, recording the absorbance at regular time intervals were all carried out separately for three times.

**Morphology Characterization.** The surface morphologies of glass microspheres without and with β-glucosidase immobilization were examined by using scanning electron microscopy (SEM) at an accelerating voltage of 15 kV (Model: JSM-6510LV, JEOL; Tokyo, Japan). Before measurements, the samples were coated with a thin layer of gold.

**Isoflavone Extraction.** The methods for extraction of isoflavone in black soymilk were in accordance with the procedures described previously.[28] Glass microsphere enzymatic systems were established in three different batch reactors under the same experimental conditions. Fifty milliliters of black soymilk was used as the substrate in each batch reactor during the deglycosylation of isoflavone. At regular time intervals, 0.75 mL of black soymilk was withdrawn from each batch reactor and added into 0.75 mL of MeOH (100%) with 533.2 ppm benzoic acid as internal standard. The volume ratio of black soymilk and MeOH was 1:1 to make the percentage of MeOH become 50%. The mixture was shaken for 3 min and centrifuged at 8000g for 1 min to remove the insoluble debris. The filtrate was collected and analyzed by HPLC. The amount of isoflavones in black soymilk (daidzin, genistin, daidzein, and genistein) was calculated by the following equation:

$$\text{Isoflavone} = \frac{A_S}{A_{I.S.}} \times \frac{C_{I.S.}}{RRF} \times \frac{1}{M.W.} \times \frac{V_{\text{Initial}}}{V_{\text{Withdrawn}}}$$

Where $A_S$ and $A_{I.S.}$ represent the peak areas of each isoflavone species and internal standard, respectively; $C_{I.S.}$ stands for the concentration of internal standard (533.2 ppm); relative response factor (RRF) for each isoflavone species that were determined to be daidzin (6.55), genistin (12.89), daidzein (14.51), and genistein (18.18); *M.W.* is the molecular weight of each isoflavone species; $V_{\text{Initial}}$ and $V_{\text{Withdrawn}}$ represent the initial volume of black soymilk (50 mL) and the withdrawn volume of black soymilk (0.75 mL), respectively.

**HPLC Analysis.** Resolution of isoflavone glycosides and aglycones was performed using YMC-Pack ODS-AMC C18 column (5 μm, 250 × 4.6 mm). The column was attached to a high performance liquid chromatography (HPLC) workstation containing two LDC pumps (constaMetric 3200, constaMetric 3500) and a LDC analytical mixer. The injection volume was 20 μL and an elution rate of 1.0 mL/min was utilized. Acetic acid (0.1%) in aqueous solution (solvent A) and 0.1% acetic acid in acetonitrile (solvent B) were used as the eluent. Detection was performed by UV absorption at 254 nm. The data were analyzed by SISC Chromatography data system (SISC, Taiwan).

## ■ RESULTS AND DISCUSSION

**Selection of Immobilized Enzyme System.** To select the most efficient immobilized enzyme system for the isoflavone deglycosylation in soymilk, $p$NPG was used as a substrate to measure the enzyme activity. Figure 1 summarizes the catalytic
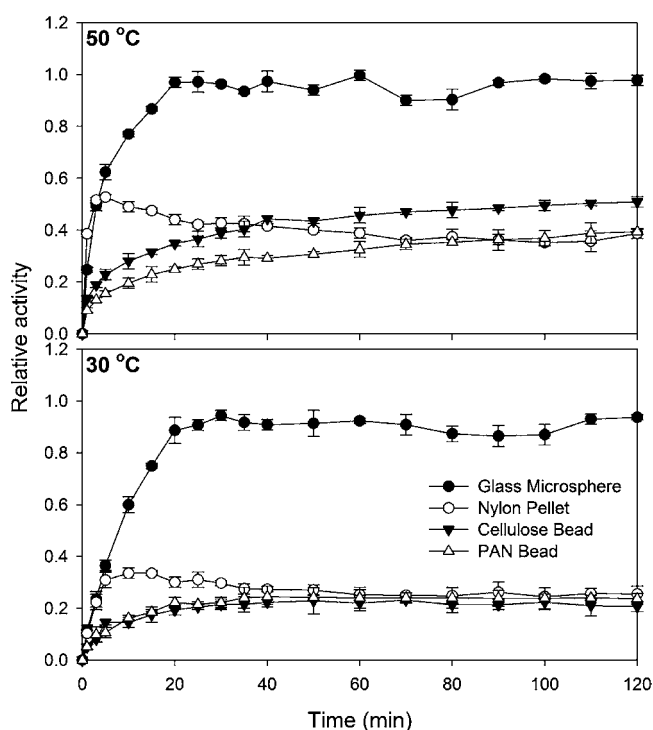


**Figure 1.** Catalytic behaviors of the immobilized $\beta$-glucosidase system using four different carriers (glass microsphere, nylon pellets, cellulose beads, and PAN beads) at 30 and 50 °C, respectively. The amount of $\beta$-glucosidase immobilized on each type of carrier was measured as follows: $42.34 \pm 3.65$ mg on glass microspheres, $49.23 \pm 0.89$ mg on nylon pellets, $38.63 \pm 4.80$ mg on cellulose beads, and $32.25 \pm 0.21$ mg on PAN beads. All the experiments were performed in triplicates. The relative activity was defined as the ratio of $Abs(t)/Abs(S)$, where $Abs(t)$ represents the absorbance of the $p$NPG solution after $t$ min of $\beta$-glucosidase treatment and $Abs(S)$ stands for the absorbance of $p$-nitrophenol converted completely from $p$NPG in the solution.

performance of various carriers at two distinct temperatures. Obviously, $\beta$-glucosidase immobilized on the glass microspheres at both 30 and 50 °C exhibited the highest relative activity (30 °C, 0.94; 50 °C, 0.98) over those on the nylon pellets (30 °C, 0.26; 50 °C, 0.39), cellulose beads (30 °C, 0.21; 50 °C, 0.51), and PAN beads (30 °C, 0.24; 50 °C, 0.39). Significant differences in relative activity between glass microspheres and other three solid carriers (nylon pellets, cellulose beads, and PAN beads) occurred with the catalytic duration of more than 10 min ($p < 0.05$), which directly evidence the most effective catalytic activity of the glass microsphere enzymatic system. A saturated conversion of $p$NPG to $p$-nitrophenol by glass microspheres was achieved in 30 min.

It was suggested that the particle size of the carrier is a significant factor to influence the catalytic activity in an immobilized enzyme system.[29] Enzymes immobilized on smaller size beads exhibit higher catalytic activity due to the increased surface area and reduced substrate transfer resistance.[30,31] Among the four carriers evaluated in this study, the particle size of glass microspheres was determined to be ~10 $\mu$m diameter, which is 100 times less than that of other carriers (~1 mm), resulting in a significant differences of catalytic behavior between glass microspheres and other carriers (Figure 1). Glass microspheres with the smallest particle size exhibited the best performance (relative activity ~0.98) among the four solid carriers.

**Morphology of Glass Microspheres.** To develop an effective deglycosylation of isoflavones in soymilk, the utilization of glass microspheres as $\beta$-glucosidase carrier was adopted for further study. The scanning electronic microscope (SEM) images of glass microspheres with and without enzyme immobilization are presented in Figure 2. Prior to the enzyme attachment, the surfaces of these glass microspheres were smooth, with a diameter of ~10 $\mu$m (Figure 2A). After enzyme immobilization through covalent binding, the surfaces of glass microspheres became granulated (Figure 2B). The granulated areas marked by red arrows represent the regions of enzyme attachment, which provided direct evidence that $\beta$-glucosidase was successfully immobilized on the glass microspheres by chemical modification.

**Determination of Optimal Temperature for Free and Immobilized $\beta$-Glucosidase.** The effect of temperature on the catalytic activity of free and immobilized $\beta$-glucosidase was studied in the range of 30−80 °C. Figure 3A refers to the results of temperature dependence for free and immobilized enzyme. It is obvious that the optimum temperature was at 40 °C for immobilized enzyme and at 60 °C for free enzyme. A dramatic decrease in relative enzyme activity was observed in both systems when temperature went above 60 °C (from 1.00 to 0.14 for free enzyme; from 0.92 to 0.11 for immobilized system). Since the most suitable pH value for black soymilk was pH 6 to avoid the acid-induced aggregation,[32] the following validation experiments were carried out under the optimum conditions of 40 °C and pH 6.

**Determination of Kinetic Parameters for the Free Enzyme System and the Glass Microsphere Enzymatic System.** As shown in Figure 3B, the catalytic activity of the free $\beta$-glucosidase system and the immobilized $\beta$-glucosidase system (initial and stabilized behaviors) was recorded as function of the reaction time to determine the kinetic parameters for each case. The experimental points were fitted well by an exponential curve with an equation of $Abs(t) = Abs(S)(1 - e^{-Kt})$, where $K$ is a rate constant measured in min$^{-1}$, $\tau_{50}$ represents the time required to reach a 50% of reduction from $p$NPG to $p$-nitrophenol and $\tau_{complete}$ denotes the time necessary for the complete catalytic conversion.

The kinetic parameters were summarized in Table 1. It is obvious that the $K$ value of the immobilized enzyme system at initial behavior ($0.17 \pm 0.0$ min$^{-1}$) was larger than that of the free enzyme system ($0.09 \pm 0.0$ min$^{-1}$), which indicates the immobilized enzyme system behaved more effectively than the free enzyme system did. Temperature plays an important role in catalytic efficiency. In this respect, a significant difference in relative activity at 40 °C between free enzyme and immobilized enzyme system was clearly observed ($p < 0.05$). The relative activity of the free enzyme system at 40 °C presents only 0.5-fold lower comparing with the immobilized enzyme system (Figure 3A).
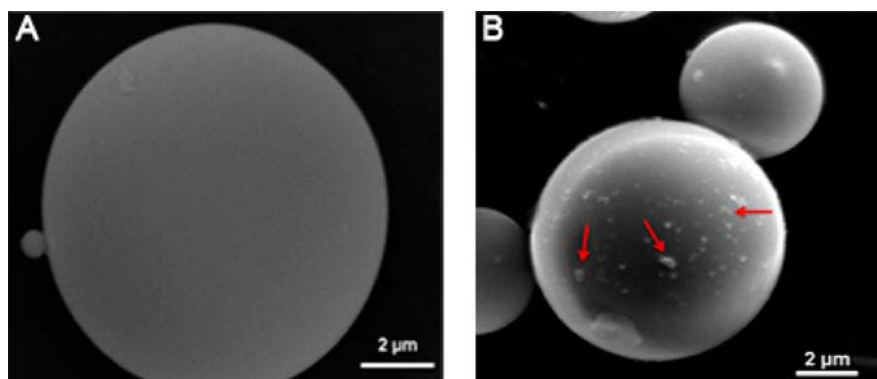
**Figure 2.** SEM images of surface morphologies of glass microspheres (A) without and (B) with $\beta$-glucosidase immobilization. The areas marked by red arrows indicate the regions of enzyme attachment by covalent bonding.
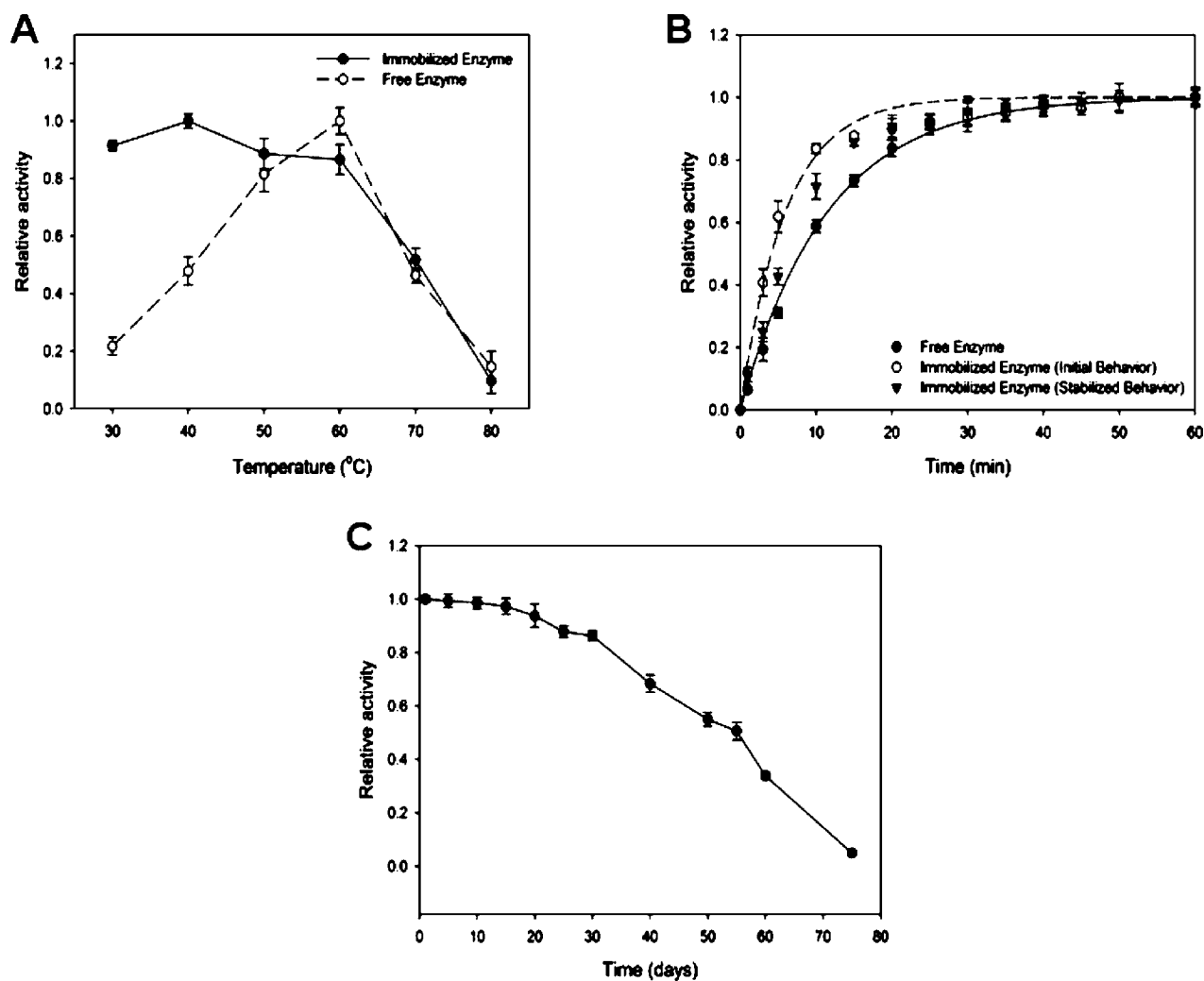


**Figure 3.** (A) Effect of temperature on the activity of free $\beta$-glucosidase and immobilized $\beta$-glucosidase at pH 6. (B) The batch reaction of free enzyme system and the recycling batch reactions of the glass microsphere enzymatic system and their kinetic parameters determination by fitting curves. (C) Storage stability of the glass microsphere enzymatic system recorded from 1 to 75 days. All the experiments were performed in triplicates.

On the other hand, the immobilized enzyme system at initial behavior ($0.17 \pm 0.01$ min$^{-1}$) exhibited a higher $K$ value in comparison to the immobilized enzyme system at stabilized behavior ($0.12 \pm 0.01$ min$^{-1}$). This phenomenon is common because the attachment of some enzymes onto the supports was through van der Waal's forces or ion—ion interaction instead of covalent binding. $\beta$-Glucosidase with amino acids reacted with glutaraldehyde mainly by $-C\!=\!N-$ bonds, which leads to an attachment through covalent binding between enzymes and solid carriers. However, the functional group of the solid carriers sometimes may not be entirely responsible for the chemical modification. For example, the amino acid of enzyme with hydroxyl, carboxylic, and amine groups may interact with $-OH$ groups on the surface of glass microspheres or cellulose beads

**Table 1. Kinetic Parameters Characterizing the Free Enzyme System and the Glass Microsphere Enzymatic System**[a]

|  | free enzyme | immobilized enzyme (initial behavior) | immobilized enzyme (stabilized behavior) |
|---|---|---|---|
| $K$ (min$^{-1}$) | $0.09 \pm 0.01$ | $0.17 \pm 0.01$ | $0.12 \pm 0.01$ |
| $\tau_{50}$ (min) | $7.92 \pm 0.11$ | $4.12 \pm 0.08$ | $5.95 \pm 0.16$ |
| $\tau_{complete}$ (min) | 30 | 30 | 30 |

[a]The experiments were performed in triplicates.

through hydrogen bonding instead of covalent binding. The situation may lead to a leakage between enzyme and support,[22] which results in a resuspension of unbound enzymes in solution;

nevertheless, the suspended enzymes are still involved in the catalytic performance during the first few cycles. Our results were consistent with this phenomenon. In the case of the immobilized enzyme system, catalytic capability of batch reactions remained consistent after 40 cycles of use (data not shown).

**Storage Stability of the Glass Microsphere Enzymatic System.** The storage stability of the $\beta$-glucosidase immobilized system from 1 to 75 days is shown in Figure 3C. It was found that the glass microsphere enzymatic system retained 95% of its original activity after 30 days. The relative catalytic activity remained above 0.8 even after 40 days. A dramatic decrease of relative activity was observed after 75 days (0.22). The results
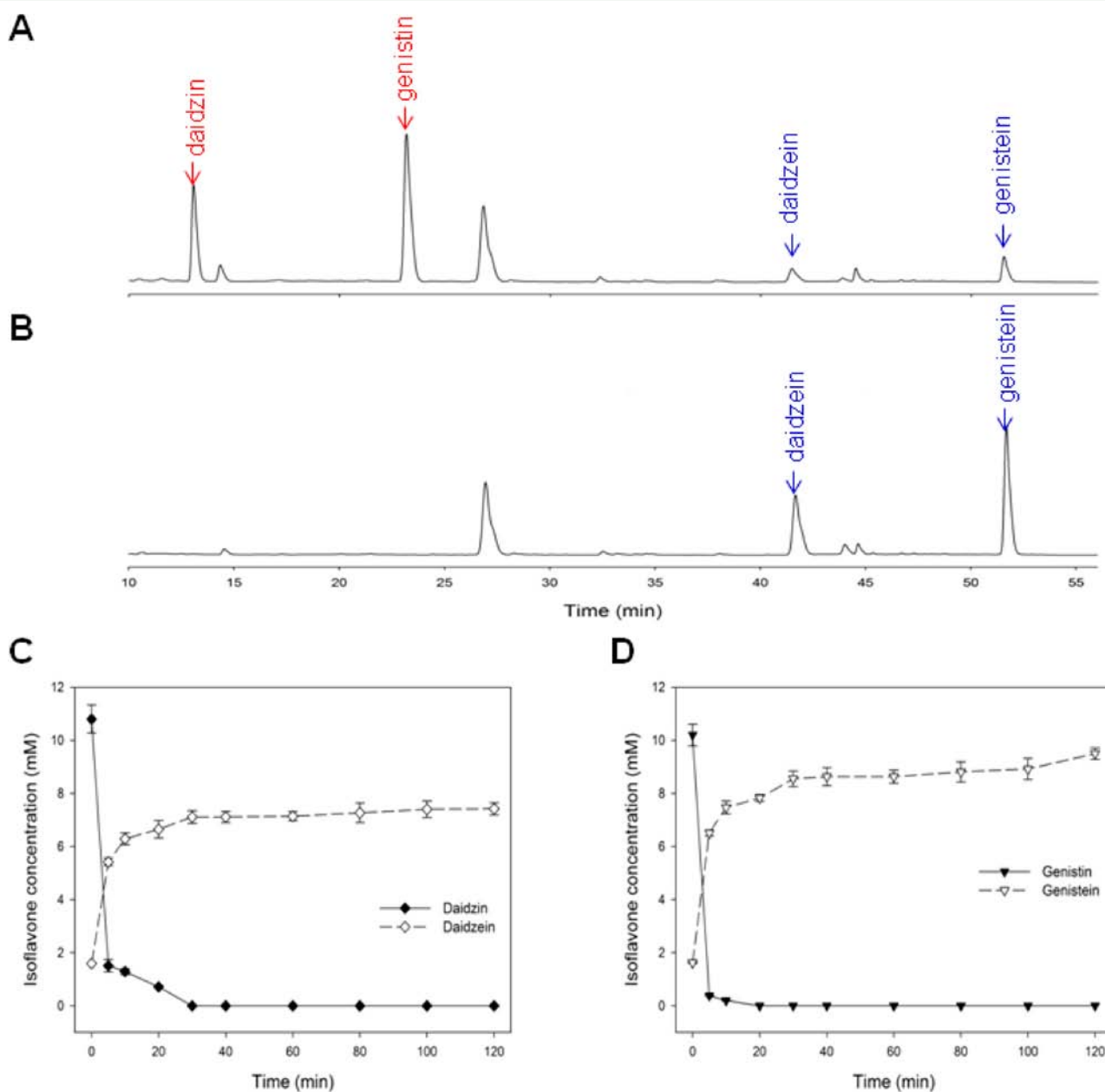


**Figure 4.** HPLC chromatograms of isoflavones in black soymilk (A) without any treatments by the glass microsphere enzymatic system; (B) treated by the glass microsphere enzymatic system for 30 min. The retention time of isoflavone glycosides (daidzin and genistin) was 13 and 23 min, respectively. Isoflavone aglycones, daidzein and genistein, were clearly the more slowly moving compounds, eluting apart at 40 and 51 min. (C) Conversion of daidzin to daidzein in black soymilk by the glass microsphere enzymatic system. (D) Conversion of genistin to genistein in black soymilk by the glass microsphere enzymatic system. All the experiments were performed in triplicates.

**Table 2. Isoflavone Contents of Black Soymilk Treated at Various Reaction Times by the Glass Microsphere Enzymatic System[a]**

| reaction time | glycoside (mM) | | aglycone (mM) | | aglycosylation rate (%)[b] | | aglycone (%) in the total isoflavones[c] |
| --- | --- | --- | --- | --- | --- | --- | --- |
| | daidzin | genistin | daidzein | genistein | daidzin | genistin | |
| Nontreated | $10.80 \pm 0.53$ | $10.20 \pm 0.41$ | $1.59 \pm 0.03$ | $1.62 \pm 0.03$ | – | – | $13.26 \pm 0.29$ |
| 10 min | $1.29 \pm 0.11$ | $0.20 \pm 0.01$ | $6.29 \pm 0.22$ | $7.47 \pm 0.24$ | $49.42 \pm 0.38$ | $58.50 \pm 0.23$ | $56.84 \pm 0.43$ |
| 30 min | 0 | 0 | $7.11 \pm 0.24$ | $8.55 \pm 0.29$ | $51.11 \pm 0.54$ | $67.94 \pm 0.17$ | $64.68 \pm 0.46$ |
| 60 min | 0 | 0 | $7.14 \pm 0.17$ | $8.63 \pm 0.25$ | $51.39 \pm 1.17$ | $68.73 \pm 0.59$ | $65.14 \pm 0.92$ |
| 120 min | 0 | 0 | $7.42 \pm 0.23$ | $9.50 \pm 0.21$ | $53.98 \pm 0.76$ | $77.25 \pm 1.28$ | $69.89 \pm 1.03$ |

[a]The experiments were performed in triplicates. [b]Aglycosylation rate (%) = (increased molarity of isoflavone aglycones/decreased molarity of corresponding glycosylated isoflavone) × 100%. [c]Aglycone (%) in the total isoflavones = (the amount of aglycones/the total amount of initial isoflavones) × 100%.

**Table 3. Kinetic Parameters of the Glass Microsphere Enzymatic System for Isoflaovones in Black Soymilk[a]**

| | daidzin | daidzein | genistin | genistein |
| --- | --- | --- | --- | --- |
| $K$ (min$^{-1}$) | $0.35 \pm 0.04$ | $0.21 \pm 0.02$ | $0.65 \pm 0.03$ | $0.20 \pm 0.03$ |
| $\tau_{50}$ (min) | $2.04 \pm 0.25$ | $1.23 \pm 0.15$ | $1.19 \pm 0.08$ | $1.87 \pm 0.22$ |
| $\tau_{complete}$ (min) | 30 | 30 | 20 | 30 |

[a]The experiments were performed in triplicates.

indicated that the storage capacity of the $\beta$-glucosidase immobilized system was around 40 days.

**Hydrolysis of Isoflavone Glycosides in Black Soymilk to Isoflavone Aglycones.** The catalytic conversion from isoflavone glycosides into isoflavone aglycones was performed using 50 mL of black soymilk as substrate. Obviously, the relative intensity of glycosides was significant higher than that of aglycones in the nontreated black soymilk (Figure 4A). However, after treatment with the glass microsphere enzymatic system for 30 min, the peaks of glycosides disappeared, whereas an enhancement of relative intensity of aglycones was observed (Figure 4B), which indicates that the entire deglycosylation of isoflavone was achieved by the immobilized $\beta$-glucosidase system. Isoflavone content for different experimental conditions is summarized in Table 2. It is obvious that the amount of isoflavone glycosides reduced significantly from 21 to 1.49 mM in 10 min, meanwhile, the amount of aglycones increased from 3.21 to 13.76 mM, which reveals that the strategy of enzyme immobilization on glass microspheres was successful in hydrolysis of isoflavone in black soymilk. The complete deglycosylation of both daidzin and genistin by the glass microsphere enzymatic system occurred in 30 min (Figure 4C,D). An enrichment of the aglycone content in the total isoflavones in black soymilk was observed by 51.42% under a 30 min treatment by the glass microsphere enzymatic system (Table 2). Moreover, the kinetic parameters for isoflavone glycosides and aglycones, including rate constant ($K$) at which isoflavone glycosides deglycosylation were determined, the time ($\tau_{50}$) in which 50% of isoflavone glycosides deglycosylation was reached, and the time ($\tau_{complete}$) required to achieve complete isoflavone glycosides deglycosylation, have further been investigated and summarized in Table 3. The rate constant ($K$) for genistin (0.65 $\pm$ 0.0 min$^{-1}$) was higher than that for daidzin (0.35 $\pm$ 0.0 min$^{-1}$), which reveals that the glass microsphere enzymatic system deglycosylated genistin faster than daidzin. The almost equal $K$ values between daidzein (0.21 $\pm$ 0.02 min$^{-1}$) and genistein (0.20 $\pm$ 0.03 min$^{-1}$) reflect that the enhancement of two aglycones by glass microsphere enzymatic system was in an equivalent rate. $\tau_{complete}$ for daidzin, genistin, daidzein, and genistein was determined to be 30, 20, 30, and 30 min, respectively. In our previous studies, the microorganism fermentation system took 1 to 2 days to reach a complete deglycosylation.[17,33] Our novel enzyme immobilization system, in this regard, accomplished the highest yield of isoflavone aglycones in only 30 min, which demonstrated that this enzymatic system is a remarkable method for the scale-up preparation of aglycones-rich products.

Recently, some studies reported that the utilization of thermostable $\beta$-glucosidase isolated from some microorganisms is a potential approach for deglycosylation of isoflavone glycosides.[34−37] However, suspended enzymes without any supports can only perform single catalysis due to the difficulty in recovering enzymes from products. In the present work, we report the covalent immobilization of $\beta$-glucosidase onto four different solid carriers (glass microspheres, nylon pellets, cellulose beads, and PAN beads). Taking advantage of the smallest particle size, glass microspheres sustained the enzyme most effectively and were chosen for the enrichment of isoflavone aglycones in soymilk. The reaction time for complete deglycosylation in this study required only 30 min, which is more time-saving in comparison to at least 120 min by suspended $\beta$-glucosidase.[34−36] Moreover, the capacity of multiple reusability (40 times) and the long-term storage (stable for 40 days) enable the glass microsphere enzymatic system as a potential platform for further scale-up industrial applications.

In conclusion, $\beta$-glucosidase immobilized on glass microspheres is capable of catalyzing the hydrolysis of isoflavone glycosides of black soymilk in a highly efficient catalytic manner over other three solid carriers (nylon pellets, cellulose beads, and PAN beads). The benefits of 40 consecutive reuses and at least 40-days stability raise the value of this immobilized enzyme system, which can potentially act as a replacement for free $\beta$-glucosidase to deglycosylate isoflavone glycosides in black soymilk. In addition, the adoption of the low-cost solid carrier (i.e., glass microspheres) as enzyme immobilized support provides an economic way to prepare the aglycone-rich black soymilk. Moreover, for cost-saving purposes, the replacement of commercialized $\beta$-glucosidase with cloning and expression of $\beta$-glucosidase on a large scale using molecular techniques will be the next challenge.

## ■ AUTHOR INFORMATION

### Corresponding Author
*Tel: +886-2-33661502. Fax: +886-2-23620849. E-mail: kccheng@ntu.edu.tw.

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