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RESEARCH

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Innovative approach for semi-continuous production of puerarin palmitate via transesterification with novel immobilized lipase

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Abstract

Background Puerarin, a flavonoid abundantly found in the roots of *Pueraria lobata*, exhibits antioxidant, anti-cancer, anti-inflammatory, and anti-aging properties. Despite its numerous benefits, the low bioavailability of puerarin hinders its widespread industrial application. To improve this, flavonoid acylation is required. Lipase is a biocatalyst primarily used for flavonoid acylation; immobilized lipase is preferred given the limited stability of free lipase. Although research on flavonoid acylation for structural modification is actively being conducted, there is a need to explore continuous production for enhancing the industrial application of flavonoid esters. Therefore, in this study, we aimed to produce puerarin palmitate innovatively by selecting an effective lipase, developing an immobilized lipase, characterizing the enzymatic reaction, and designing a semi-continuous reactor system.

Results Lipase from *Thermomyces lanuginosus* (TL) was found to be the most suitable for the synthesis of puerarin palmitate; it was successfully immobilized on glutaraldehyde-activated silica gel (GASG). TL GASG showed greater solvent, thermal, and operational stability than those of commercial immobilized lipases. Furthermore, TL GASG use resulted in a puerarin palmitate conversion rate of 97.17% within 3 h. Additionally, the reusability of TL GASG for this reaction was higher than that of commercially available immobilized lipases. Upon using a semi-continuous reactor, the cumulative conversion rate of puerarin palmitate remained above 85% after more than ten substrate injections.

Conclusions The results support the feasibility of the continuous production of flavonoid esters, underscoring their diverse industrial applications. A biorefinery strategy is anticipated to be proposed to utilize *P. lobata* extract biomass for flavonoid ester production. Moreover, optimizing continuous reactors through statistical experimental design is expected to enhance flavonoid ester production efficiency; these aspects will be explored in future research.

Highlights

- The stability of TL GASG was higher than commercial immobilized lipases.
- The novel immobilized lipase was applied to puerarin ester synthesis.

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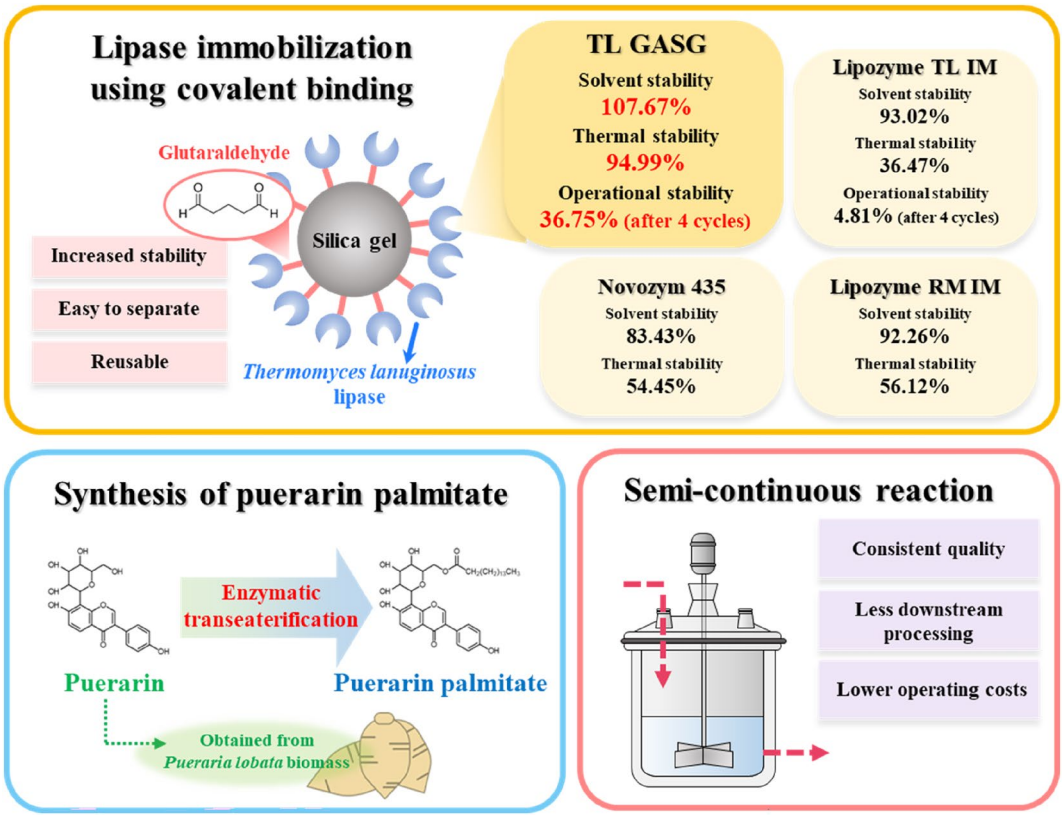
- The maximum conversion of puerine palmitate was achieved 97.17% in 3 h.
- Conversion rate via semi-continuous reaction was maintained > 85% after 10 injections.

Keywords Lipase, Immobilization, Flavonoid, Puerarin, *Pueraria lobata*, Acylation, Semi-continuous reaction

Graphical Abstract



Introduction

Flavonoids are polyphenol compounds found in plants; more than 8,000 flavonoids have been identified to date [1]. They have various biological properties such as antioxidant [2–5], anti-inflammatory [6, 7], anti-cancer [8, 9], and anti-aging [8] effects, and research is being actively conducted to apply them in various industries such as food, medicine, and cosmetics [10–12]. The basic structure of a flavonoid comprises a C6-C3-C6 carbon skeleton with two benzene rings connected to a heterocyclic pyrene ring. Flavonoids are classified into the following six classes according to the degree of oxidation of the heterocyclic ring and the number of substituents on the hydroxyl or methyl group of the benzene ring: flavanones, flavones, flavonols, isoflavones, flavanols, and anthocyanins [10].

Puerarin (daidzein-8-C-glucoside) belongs to the isoflavone class and is abundant in the roots of *Pueraria lobata,* which is mainly cultivated in Asia, including China, Japan, and Korea. *P. lobata* is rich in various bioactive substances, primarily puerarin, and is widely used as a medicinal and edible ingredient [13, 14]. Puerarin exhibits various biological activities, such as estrogen-like activity, vasodilation, cardioprotection, blood glucose reduction, neuroprotection, reduction in the levels of alcohol intake, and antioxidant, anti-inflammatory, anti-cancer, and anti-aging effects [14, 15].

Despite these known diverse benefits, puerarin is currently only available in limited forms, primarily as intravenous injections or eye drops [16, 17], because the oral bioavailability of flavonoids is only ~ 7% due to their low lipid solubility and stability [2, 18]. Puerarin belongs to

the class IV category with very low lipid solubility and low permeability in the biopharmaceutical classification system because it has many hydroxyl groups in its molecular structure [19]. Moreover, the large number of hydroxyl groups in puerarin reduces metabolic stability, preventing it from exerting its full efficacy [14]. Its limited bioavailability hinders widespread utilization in diverse industries, including clinical trials, food, and cosmetics, necessitating enhancement [18].

To overcome these limitations, research on flavonoid ester synthesis via acylation is being actively conducted [20–22]. Flavonoid esters synthesized through acylation show an increased cell delivery rate due to improved lipid solubility [14]. In addition, the stability is improved owing to the substituted acyl group; hence they can be exerted without losing their biological activity during metabolic activity [14]. Additionally, improving the lipophilicity of flavonoid esters is advantageous for their application in food matrixes, facilitating their use in the food industry [23]. In a previous study, we synthesized a naringin ester through enzymatic acylation to enhance the lipid solubility of naringin [3, 4]. Xiao et al. employed acylation to synthesize troxerutin fatty acid esters using a fatty acid vinyl ester to improve lipid solubility [24]. Mo et al. synthesized puerarin esters using acyl donors with chain lengths of C3–C14; they reported that the stability of these esters in digestive juices was improved and that the stability increased with an increase in the chain length of the acyl donor [14]. Notably, the synthesis of puerarin esters using acyl donors with longer chain lengths has not yet been reported. In our previous study, we selected a C16 acyl donor to synthesize a puerarin ester from a C12–C18 long-chain acyl donor [2].

Recently, lipase, a biocatalyst, has emerged as the preferred enzyme for flavonoid acylation. Lipase-mediated biocatalysis offers several advantages over conventional chemical catalysts, including regioselectivity, non-toxicity, and biodegradability [20]. Despite these advantages, free lipases obtained from nature have limitations such as relatively low stability, high costs, and difficulty in their recovery. To overcome these limitations, enzyme immobilization, wherein free lipase is immobilized on an insoluble support, has been proposed [25–27]. Immobilized enzymes offer enhanced stability, facile recovery, and reusability, making them conducive for continuous reaction applications [28].

Enzyme immobilization can be broadly categorized into physical and chemical methods. Physical immobilization methods include adsorption, entrapment, and encapsulation, whereas chemical methods involve covalent binding and cross-linking [28]. Commercially available immobilized enzymes include Novozym 435, Lipozyme TL IM, and Lipozyme RM IM, all of which are manufactured via physical adsorption [29, 30]. When enzymes are immobilized using the physical adsorption method, they may be released from the support via exposure to high temperatures, organic solvents, or surfactants such as free fatty acids [28]. When an enzyme is released from the support, the functional stability of the immobilized enzyme deteriorates, controlling the reaction conditions becomes difficult, and subsequent separation and purification processes become highly complex. Covalent binding using glutaraldehyde as a linker does not cause enzyme loss owing to the abovementioned factors, allowing it to perform properly as an immobilized enzyme [28]. Therefore, developing an immobilized enzyme using the covalent binding method is necessary to extend the application of puerarin palmitate synthesis reactions from batch to continuous systems.

Immobilized enzymes with enhanced operational stability can also be applied in continuous reactors, offering the advantages of maintaining consistent quality, streamlining downstream processes, and reducing capital and operating costs [31, 32]. For instance, Nie et al. continuously produced biodiesel using Novozym 435 in a fixed-bed reactor [33]. Similarly, Ubilla et al. continuously produced lactulose from fructose and lactose using immobilized enzymes in a continuously stirred tank reactor [34]. However, only one instance of continuous production of a flavonoid ester has been reported, wherein Du et al. synthesized neohesperidin esters using a microfluidic reactor [22]. Given the limited research on the continuous production of flavonoid esters, there is a need for more active investigation to enhance the practicality of the flavonoid ester production process.

In this study, we aimed to develop a novel immobilized lipase and apply a semi-continuous reaction to efficiently synthesize puerarin palmitate. In particular, to improve the lipid solubility of puerarin, we applied a transesterification reaction with a longer C16 acyl donor, which has not been reported before. To our knowledge, this innovative approach has been implemented for the first time in the field of flavonoid ester synthesis. To achieve this, a lipase suitable for the synthesis of puerarin palmitate was selected and the stability of the immobilized enzyme prepared using this lipase was evaluated. In addition, the enzymatic conversion of puerarin palmitate using the novel immobilized lipase was performed in batch and semi-continuous modes. The findings of this study would facilitate the continuous production of flavonoid esters and their utilization in diverse industrial applications.

Materials and methods

Materials

Free lipases—Lipozyme CAL B L (*Candida antarctica* lipase B (CAL B)), Lipozyme TL 100 L (*Thermomyces*

lanuginosus lipase (TL lipase)), and Palatase 20000 L (*Rhizomucor miehei* lipase (RM lipase))— and three commercial immobilized lipases, namely Novozym 435 (CAL B immobilized on an acrylic resin), Lipozyme TL IM (TL lipase immobilized on a silica gel carrier), and Lipozyme RM IM (RM lipase immobilized on a resin carrier) were purchased from Novozymes (Bagsvaerd, Denmark). Silica gel, which was used as a support, was purchased from Grace Davison (Columbia, MD, USA). *p*-Nitrophenyl palmitate (*p*-NPP; 98%), for measuring lipase activity and (3-aminopropyl)triethoxysilane (3-APTES; 99%), and glutaraldehyde (25% in H_2O, grade II), for surface modification, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Puerarin (99%) was purchased from Alibaba Co. Ltd. (Hangzhou, China). *n*-hexane (96%, guaranteed reagent) was purchased from Junsei (Tokyo, Japan), while *tert*-amyl alcohol (99%, guaranteed reagent), acetone (99.5%, extra pure), and hydrogen peroxide (30–35%, extra pure) were purchased from Daejung (Gyunggido, Korea). *p*-Nitrophenol (99%), palmitic anhydride (96%), and vinyl palmitate (96%) were purchased from TCI (Tokyo, Japan). High-performance liquid chromatography (HPLC)-grade acetic acid (99.7%), methanol (99.8%), and water, which were used as mobile phases, were purchased from J.T. Baker (Philipsburg, NJ, USA).

Evaluation of enzyme stability based on lipase activity

p-NPP assay

Lipase activity was determined using the *p*-nitrophenyl palmitate (*p*-NPP) assay to select lipase based on solvent and thermal stability of lipase and evaluate the operational stability of the immobilized lipase [35]. Briefly, *p*-NPP (10 mM) in acetonitrile was mixed with lipase suspended in 50 mM Tris–HCl buffer (pH 8.0) in a 1:1 (v/v) ratio. Subsequently, the reaction was terminated by incubating the solution in boiling water for 5 min. The reaction mix was centrifuged, following which the supernatant was diluted 10 times with distilled water. Then, the number of moles of *p*-nitrophenol (*p*-NP) produced was calculated by measuring the absorbance at 410 nm using a UV/Vis spectrophotometer. One unit was defined as the number of moles of *p*-NP produced by 1 mL lipase in 1 min. The lipase activity of the immobilized enzyme was measured in the same manner, but the enzyme was filtered after the reaction to terminate it. All experiments were performed at least in duplicate; results are presented as the average of the values.

Solvent stability of the lipases

Solvent stability of the lipases was investigated as follows. Lipase was prepared by suspending it in a 50 mM Tris-HCl buffer (pH 8.0), mixing it with the solvent at a 1:3 (v/v) ratio, and incubating it for 72 h at 4 °C to minimize the thermal effects. Enzyme activity was measured via the *p*-NPP assay using the incubated solution diluted five times with the buffer. As a control, the lipase suspension and solvent were mixed at a 1:3 (v/v) ratio under the same conditions as described above. However, enzyme activity was measured by immediately diluting the solution with five volumes of the buffer without incubation. The residual lipase activity concerning solvent stability was then calculated relative to the activity of the control.

Thermal stability of the lipases

The thermal stability of the lipases was investigated using the following procedure. Lipase was prepared by suspending it in a 50 mM Tris-HCl buffer (pH 8.0) and subsequently incubating it at various temperatures for 1 h. The activity was determined via the *p*-NPP assay using the lipase suspension post incubation. A lipase suspension that was not incubated was used as the control. The residual lipase activity concerning thermal stability was then calculated relative to the activity of the control.

Operational stability of the immobilized lipases

Next, the operational stability of the immobilized lipase was investigated. Briefly, 10 mg of immobilized lipase was prepared by suspending it in a buffer and then mixed with a 10 mM *p*-NPP solution at a 1:1 (v/v) ratio to assess lipase activity. After the completion of the reaction, the reaction solution was removed, and the immobilized enzyme was washed with a fresh buffer, resuspended in a fresh buffer, and used repeatedly in subsequent reactions. The residual activity concerning operational stability was calculated relative to the initial activity of the immobilized enzyme.

Lipase immobilization via covalent binding with glutaraldehyde

Lipase immobilization through covalent binding using glutaraldehyde as a linker was performed following the method described by Lee et al. [36]. Briefly, 1 g silica gel was added to 20 mL of 15% (v/v) 3-APTES in acetone and incubated at 50 °C for 2 h on a magnetic stirrer (Fig. 1a). The silica gel was washed with distilled water, dried in an oven at 60 °C for 2 h, and functionalized with amines via treatment with 3-APTES suspended in 0.05 M phosphate buffer (pH 8.0). Glutaraldehyde (preheated to 64 °C) was then added to the mixture and incubated at 4 °C for 1 h and then at 20 °C for 1 h (Fig. 1b). The resulting glutaraldehyde-activated silica gel (GASG) was washed with buffer and distilled water, dried in an oven at 70 °C for 2 h, and stored at 4 °C until use. Lipase was immobilized by adding 10 g GASG to 100 mL of a lipase suspension (8.65 mg protein/mL) in 50 mM Tris-HCl buffer (pH 8.0) and incubating the mixture at 4 °C for 24 h (Fig. 1c). The

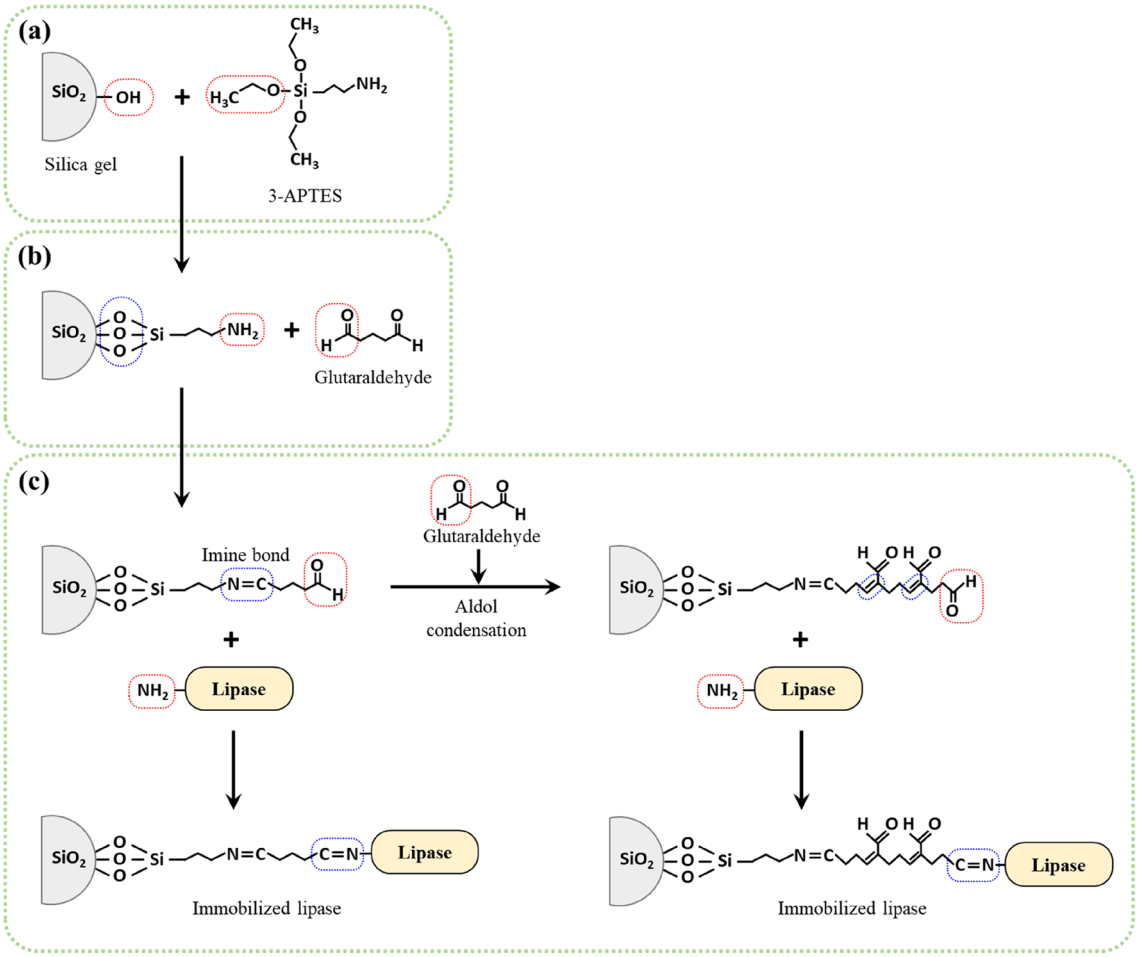
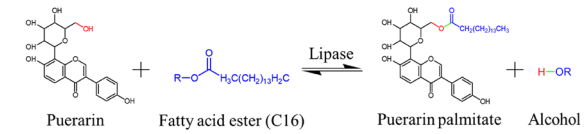


Fig. 1 Chemical strategy for lipase immobilization: covalent binding with glutaraldehyde. **a** Amino functionalization of silica gel, **b** glutaraldehyde activation of amino-functionalized silica gel, and **c** lipase immobilization on glutaraldehyde-activated silica gel



R1 = $-CO(CH_2)_{14}CH_3$, (acid anhydride)
R2 = $-CH(CH_2)$, (fatty acid vinyl ester)

Fig. 2 Reaction formula for synthesizing puerarin palmitate from puerarin and fatty acid esters via transesterification

immobilized lipase was washed with buffer and distilled water, dried at 4 °C for three days, and then sealed and stored until use.

Enzymatic synthesis of puerarin palmitate

Puerarin palmitate was synthesized through the enzymatic acylation of puerarin and two acyl donors—palmitic anhydride and vinyl palmitate (Fig. 2). First, puerarin was dried in a vacuum desiccator (Gast Manufacturing, MI, USA) with silica gel for more than seven days. Puerarin (10 mM) and the acyl donors (150 mM) dissolved in an organic solvent were then added to a 50 mL serum bottle with a working volume of 10 mL. An equal amount of immobilized lipase was added to *p*-NPP (25 U/L). The inlet of the serum bottle was then sealed to prevent the evaporation of the organic solvent. The reaction proceeded for 6 h in a shaking incubator (Jeio Tech, Daejeon, Korea) with constant stirring at 180 rpm and 40 °C. All experiments were performed in duplicate; results are presented as average values.

The experimental procedure for reusing immobilized lipase in the puerarin palmitate synthesis reaction was as follows. First, the immobilized lipase was filtered from the reaction solution using a filter paper. The immobilized lipase was then washed with *tert*-amyl alcohol, used as a reaction solvent, washed with *n*-hexane for rapid drying, and dried in a vacuum desiccator for at least 12 h. The dried immobilized enzyme was

then reused in the subsequent puerarin palmitate synthesis reaction.

Semi-continuous reaction for the synthesis of puerarin palmitate

The semi-continuous reaction was performed as follows. First, the concentration and molar ratio of puerarin to vinyl palmitate, reaction temperature, stirring speed, and reaction volume were determined under the same conditions as those for the batch reaction. Initially, for a certain period, the reaction proceeded without product recovery or substrate supply to reach a steady state. After the reaction reached a steady state, 1 mL of the reaction solution was recovered every 30 min for 300 min, and 1 mL of feed was added to the reactor. The newly supplied substrate was supplied at the same concentration as that of the initially supplied substrate. The reaction solution recovered at each time point was analyzed via HPLC, and the cumulative conversion to puerarin palmitate was calculated according to Eq. (1), as follows:

$$\text{Cumulative conversion (\%)} = \frac{[C]_{cons}}{[C]_{sup}} \times 100\,(\%) \tag{1}$$

where $[C]_{sup}$ is the cumulative supply of puerarin and $[C]_{cons}$ is the cumulative consumption of puerarin.

Analytical methods

Fourier transform-infrared (FT-IR) analysis

Lipase immobilization was analyzed using Fourier transform-infrared (FT-IR) spectroscopy (JASCO, Tokyo, Japan). TL lipase immobilized on glutaraldehyde-activated silica gel (TL GASG), glutaraldehyde-activated silica gel (GASG), silica gel treated with 3-APTES, and bare silica gel were prepared as finely ground powders after drying, and TL lipase was prepared as a solution. FT-IR analysis was conducted in attenuated total reflectance mode using a ZnSe crystal; the frequency range was set to 650–4000 cm^{-1}. All spectra were obtained in 32 scans at a 4 cm^{-1} resolution.

HPLC analysis

HPLC was used to quantitatively analyze puerarin according to the method described by Lee et al. [3]. First, an aliquot of the reaction mix was removed with a 1 mL syringe and diluted 10 times with methanol. The aliquot was filtered with a 0.2 μm syringe filter (Advantec, Tokyo, Japan) to filter out impurities and the immobilized enzyme. The filtered sample was then placed in a 2 mL HPLC vial. The samples were quantitatively analyzed using 1260 Infinity II Agilent (Santa Clara, CA, USA). Then, puerarin was detected at a wavelength of 250 nm using VWD as a detector. Puerarin was separated using an INNO Column C18 (4.6×250 mm, 5 μm) at a column temperature of 50 °C. The following mobile phases were used at a flow rate of 1 mL/min: (a) 1% acetic acid in water and (b) methanol. The gradient system was 0 min (70% A, 30% B), 5 min (0% A, 100% B), 10 min (0% A, 100% B), 15 min (70% A, 30% B), and 20 min (70% A, 30% B). A standard curve of puerarin was obtained using a sample dissolved in methanol. The conversion rate was calculated from the initial puerarin concentration and the concentration of puerarin after the reaction using Eq. (2) as follows:

$$\text{Conversion (\%)} = \frac{[C]_{\text{puerarin, initial}} - [C]_{\text{puerarin, residual}}}{[C]_{\text{puerarin, initial}}} \times 100(\%) \tag{2}$$

where $[C]_{\text{Puerarin, initial}}$ is the initial concentration of puerarin and $[C]_{\text{Puerarin, residual}}$ is the residual concentration of puerarin.

Statistical analysis

Statistical analysis was performed using Origin software with analysis of variance (ANOVA) and pairwise multiple comparisons. To evaluate the statistical significance of experimental results, we conducted one-way ANOVA and, if required, two-way ANOVA. Subsequently, we performed pairwise multiple comparisons using Tukey's honestly significant difference (HSD) test to determine whether the experimental mean values were significantly different from each other at P-value < 0.05.

Results and discussion

Lipase selection based on stability

Solvent stability of the lipases

During flavonoid ester synthesis, organic solvents shift the thermodynamic equilibrium to favor transesterification over hydrolysis. Organic solvents also affect the activity, stability, and denaturation of enzymes as well as the solubility and regioselectivity of substrates [3, 4]. In general, lipase is known to be stable in hydrophobic solvents, but it is disadvantageous for the synthesis of esters of hydrophilic hydrocarbons and sugars [4]. In our previous work, puerarin palmitate was synthesized using relatively hydrophilic organic solvents (1,4-dioxane, acetone, tetrahydrofuran, *tert*-butyl alcohol, and *tert*-amyl alcohol), and more than 95% conversion was achieved. In particular, mass spectral analysis of the reactants demonstrated that only one OH group of puerarin and the fatty acid were involved in the transesterification reaction [2]. In the present study, considering the stability of lipase, *tert*-amyl alcohol, the most hydrophobic and known green solvent [37], was selected

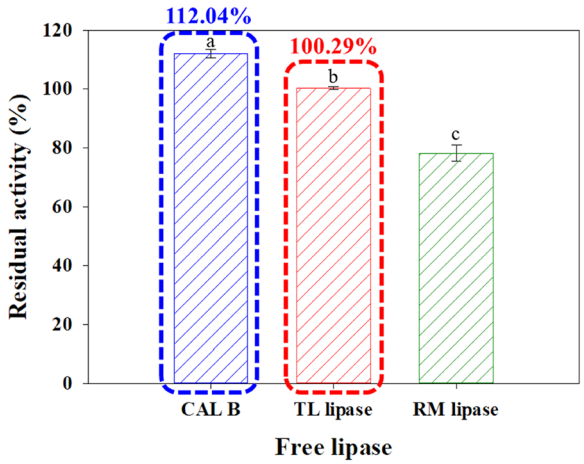


Fig. 3 Solvent stability of the three lipases in *tert*-amyl alcohol after incubation for 72 h. Reaction conditions: 10 mM *p*-NPP solution and lipase solution in 50 mM Tris-HCl buffer (pH 8.0) in a 1:1 (v/v) ratio, reaction temperature of 40 ℃, and reaction time of 20 min. Different letters above the bars indicate significant differences between lipases according to Tukey's HSD test ($P < 0.05$)

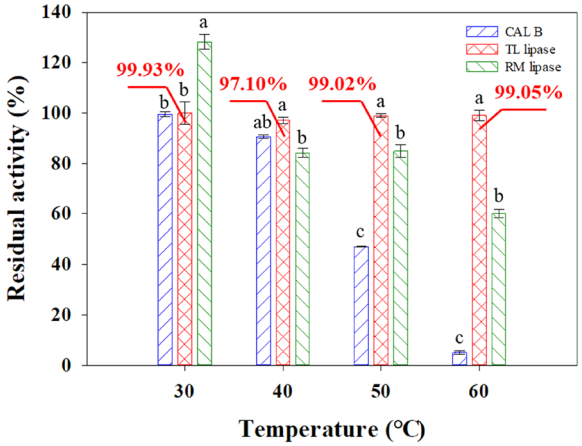


Fig. 4 Thermal stability of the three lipases after incubation at different temperatures for 1 h. Reaction conditions: 10 mM *p*-NPP solution and lipase solution in 50 mM Tris-HCl buffer (pH 8.0) in a 1:1 (v/v) ratio, reaction temperature of 40 ℃, and reaction time of 20 min. Different letters above the bars indicate significant differences between lipases at each temperature according to Tukey's HSD test ($P < 0.05$)

as the reaction solvent, and the solvent stability of three types of free lipases was investigated.

Regarding solvent stability of lipase, CAL B had the highest residual activity of 112.04 ± 1.02% followed by TL lipase at 100.29 ± 0.40%, and RM lipase at 78.16 ± 1.96% (Fig. 3). Upon performing pairwise multiple comparisons, the residual activities of the lipases were found to be significantly different. The residual activity of more than 100% observed in the CAL B and TL lipases can be attributed to hyperactivation. Certain solvents or substrates act as surfactants to increase mass transfer and stabilize the open structure of the enzyme, resulting in enzyme hyperactivation. This phenomenon is known to occur differently, depending on the type of enzyme and solvent used [38]. RM lipase showed a lower solvent stability than did the other two lipases. Lipases have a microaqueous layer necessary to maintain their activity. RM lipase is more easily stripped of its essential microaqueous layer by polar organic solvents; this tendency was also reported by Zhang et al. [39].

Thermal stability of the lipases

As the reaction temperature increases during flavonoid ester synthesis, the interaction between molecules increases, thus increasing the reaction rate. However, if the reaction temperature rises above a certain level, the enzyme gets denatured, and its activity decreases. Temperatures ranging from 30 to 60 ℃ were mainly used in previous studies on flavonoid ester synthesis, as enzyme activity tended to rapidly decrease above 60 ℃ [3, 4, 21, 22, 40, 41]. Accordingly, in the present study, we investigated the thermal stability of three types of free lipases in the temperature range of 30–60 °C.

The residual activity of RM lipase was 128.16 ± 2.06%, 84.20 ± 1.29%, 84.88 ± 1.74%, and 60.12 ± 1.26% at 30, 40, 50, and 60 ℃, respectively (Fig. 4). The residual activity of RM lipase was more than 100% at 30 ℃, which was consistent with the solvent stability result; this finding can also be attributed to hyperactivation. However, at temperatures higher than 30 ℃, the activity of RM lipase decreased, indicating low thermal stability. The residual activity of CAL B was 99.53 ± 0.67%, 90.62 ± 0.52%, 47.10 ± 0.07%, and 5.13 ± 0.55 at 30, 40, 50, and 60 ℃, respectively. Similar to RM lipase, CAL B lipase also lost its activity rapidly when exposed to temperatures ≥ 40 °C. In contrast, TL lipase maintained high residual activities of 99.93 ± 3.17%, 97.10 ± 0.88%, 99.02 ± 0.49%, and 99.05 ± 1.51% at 30, 40, 50, and 60 ℃, respectively, at the above temperatures, and there was no significant difference between them. In addition, statistical analysis using pairwise multiple comparisons showed that RM lipase had significantly higher residual activity only at 30 °C, while TL lipase exhibited significantly higher residual activity at 40–60 °C.

TL lipase is produced by the thermophilic bacterium *Thermomyces lanuginosus*. The proteins produced by thermophilic bacteria are generally stable even at high temperatures [42]. Singh et al. reported that a hemicellulase derived from *T. lanuginosus* exhibits excellent thermal stability even in the temperature range of 50–80 °C [43]. Qu et al. also reported that the thermal stability of a lipase derived from *T. lanuginosus* was superior to that of

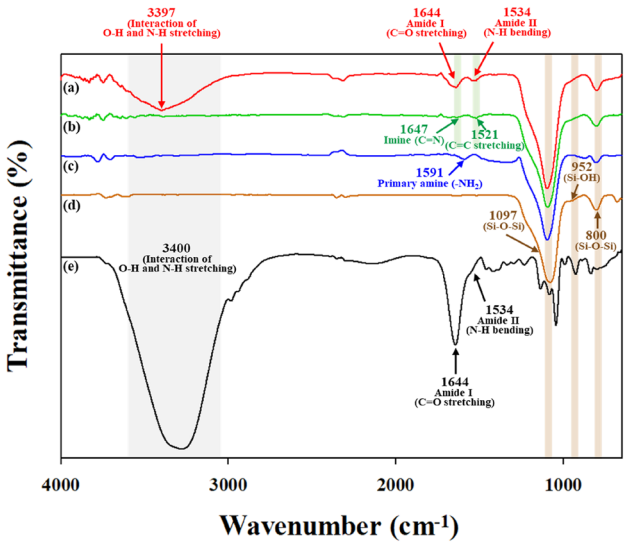


Fig. 5 Results of FT-IR analysis. **a** TL GASG, **b** GASG, **c** silica gel treated with 3-APTES, **d** silica gel, and **e** TL lipase

lipases derived from other strains [44]. Accordingly, TL lipase, which exhibits excellent stability in solvents and can stably operate over a wide range of temperatures, was selected as the lipase for immobilization.

Evaluation of the immobilized lipase

Characterization of the immobilized lipase

The immobilization yield of TL GASG achieved through enzyme immobilization was approximately 70%. The enzyme loading amount was approximately 60 mg/g matrix, similar to the results of other enzyme immobilization studies [35, 41, 45, 46]. Lipase immobilization was evaluated via FT-IR spectroscopy. Figure 5 shows the FT-IR spectra of TL GASG, GASG, silica gel treated with 3-APTES, silica gel alone, and TL lipase alone. In all the FT-IR spectra, except for those shown in Fig. 5e, the stretching vibrations of the Si–O-Si band of the silica gel were observed at 800 cm^{-1} and 1097 cm^{-1}, and the stretching vibration of the Si–OH band was observed at 952 cm^{-1} [47, 48]. As aminopropyl groups were formed on the surface of silica gel treated with 3-APTES, the band corresponding to primary amine (-NH_2) was identified, and the bending vibration of the -NH_2 band was observed at 1582 cm^{-1} (Fig. 5c) [48]. Afterward, as the aldehyde group of glutaraldehyde and -NH_2 on the surface of silica gel reacted to form an imine bond (C=N), the existing amine band faded, and a new imine band was observed at 1647 cm^{-1}. Additionally, the stretching vibration of the C=C bond generated via aldol condensation between glutaraldehyde molecules was observed at 1521 cm^{-1} [43, 48]. The spectrum of TL GASG prepared by immobilizing TL lipase on the surface of silica gel activated with glutaraldehyde is shown in Fig. 5a. The -NH_2 group of the lipase reacted with the aldehyde group to form an imine bond, and a stronger intensity of the band was observed. In addition, the C=O stretching vibration of amide I in lipase was observed at 1647 cm^{-1}, and the N–H bending vibration corresponding to amide II was observed at 1521 cm^{-1}. This was observed more strongly, as it overlapped with the imine and C=C bands present in GASG [49]. The wide band at 3400 cm^{-1}, shown in Fig. 5a and e, indicated the interactive vibrations between the O–H and N–H stretching of the lipase molecule [49]. These observations confirmed that the TL lipase was successfully immobilized on GASG.

Stability of the immobilized lipases

The stability of TL GASG in terms of solvent, temperature, and operational stability was evaluated via *p*-NPP analysis. Solvent and thermal stability were tested under the same conditions as those used for assessing free lipase stability. First, solvent and thermal stability of TL GASG was compared with that of three commercial immobilized lipases, namely Novozym 435, Lipozyme TL IM, and Lipozyme RM IM.

In terms of solvent stability, TL GASG showed the highest residual activity (107.67 ± 1.84%), followed by Lipozyme TL IM (93.02 ± 0.43%), Lipozyme RM IM (92.26 ± 1.47%), and Novozym 435 (83.43 ± 0.38%) (Fig. 6a). Pairwise comparisons revealed that TL GASG had significantly higher residual activity than the commercial lipases. Lipozyme TL IM and Lipozyme RM IM showed significantly higher residual activities than Novozym 435.

In terms of thermal stability, TL GASG maintained the highest residual activities of 101.54 ± 5.44%, 98.90 ± 3.12%, 98.73 ± 3.86%, and 94.99 ± 0.40% at 30, 40, 50, and 60 ℃, respectively (Fig. 6b). Pairwise comparisons showed that when comparing TL GASG with other commercial lipases, there was no significant difference in stability at 30 ℃, but significant differences existed at 40 and 50 ℃; at 60 °C, a highly significant difference was observed. This stability resulted from the differences in the immobilization methods. Covalent binding rigidifies the enzyme by creating a strong link between the amino acid residues of the enzyme and the support. This process helps prevent enzyme inactivation by heat and solvents, stably maintaining the three-dimensional structure of the enzyme [50]. This is the reason TL GASG showed higher solvent and thermal stability than other commercial lipases prepared via physical adsorption.

Next, the operational stability of TL GASG was evaluated as follows. After the *p*-NPP hydrolysis reaction, the immobilized enzyme was washed with buffer and used in subsequent reactions to investigate its operational

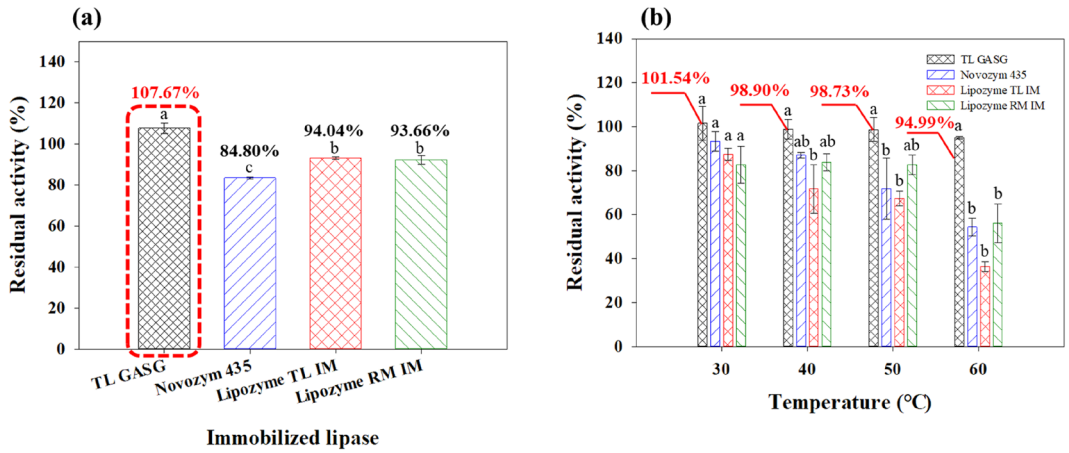


Fig. 6 **a** Solvent stability and **b** thermal stability of the immobilized lipases during *p*-NPP hydrolysis. Reaction conditions: 10–40 mg/mL immobilized enzyme, 10 mM *p*-NPP solution and lipase solution in 50 mM Tris-HCl buffer (pH 8.0) in a 1:1 ratio (v/v), reaction temperature of 50 ℃, and reaction time of 20 min. Different letters above the bars indicate significant differences between immobilized lipases according to Tukey's HSD test ($P < 0.05$)

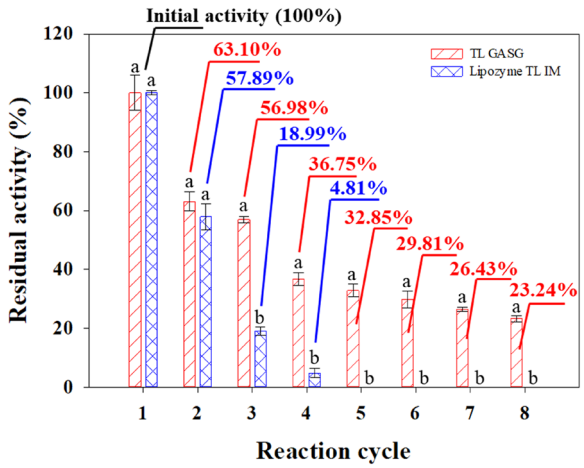


Fig. 7 Operational stability of the immobilized enzymes during *p*-NPP hydrolysis. Reaction conditions: 10–40 mg/mL immobilized enzyme, 10 mM *p*-NPP solution and lipase solution in 50 mM Tris-HCl buffer (pH 8.0) = 1:1 (v/v), reaction temperature of 50 ℃, and reaction time of 20 min. Different letters above the bars indicate significant differences between immobilized lipases at the same cycle according to Tukey's HSD test ($P < 0.05$)

stability over eight batch cycles. The operational stability of TL GASG was compared with that of Lipozyme TL IM, a commercially available immobilized lipase. Residual activities in cycles 2, 3, and 4, measured relative to the initial activity in the first cycle, were 63.10 ± 1.82%, 56.98 ± 0.64%, and 36.75 ± 1.23%, respectively, for TL GASG and 57.89 ± 2.49%, 18.99 ± 0.80%, and 4.81 ± 0.95%, respectively, for Lipozyme TL IM. Lipozyme TL IM completely lost its activity after four cycles, while TL GASG maintained residual activity of > 20% for eight cycles (Fig. 7). Multiple pairwise comparisons revealed no significant differences between the two immobilized lipases up to two cycles; nevertheless, significant differences were observed after three cycles. The rapid decrease in residual activity of TL GASG at cycle 2 is believed to be caused by the washout of less effective bound or non-covalently bound enzyme. After cycle 3, the residual activity decreases slightly overall, but it seems to be maintained by covalent bound enzyme.

The differences in the operational stability of the enzymes result from the differences in the methods used for immobilizing them. It has been reported physical adsorption causes enzymes to dissociate from the support as the reaction is repeated; hence, enzymatic activity decreases during recovery and reuse. However, covalent binding has a higher bonding strength than physical adsorption; hence, the enzymes are not easily released, maintaining their initial activity [28]. These results confirmed that TL lipase was stably immobilized on the support through covalent binding via the glutaraldehyde linker, which improved its operational stability.

Enzymatic synthesis of puerarin palmitate via transesterification with novel immobilized enzyme

Effect of the acyl donor type on the synthesis of puerarin palmitate

In our previous study, acid anhydride and fatty acid vinyl ester were found to be the most suitable acyl donors for flavonoid ester synthesis [2, 4]. In this study, a suitable acyl donor was selected for the synthesis of puerarin palmitate with TL GASG using palmitic anhydride and vinyl palmitate. It was determined from preliminary tests that

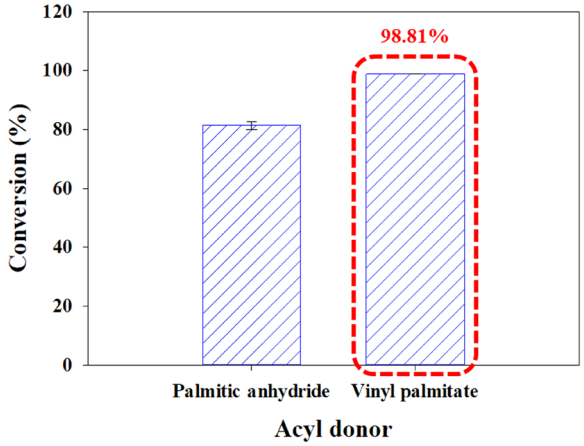


Fig. 8 Effect of the acyl donor type on the synthesis of puerarin palmitate. Reaction conditions: 25 U/L TL GASG, 1:15 molar ratio of puerarin to acyl donor, reaction temperature of 40 ℃, *tert*-amyl alcohol as the solvent, and a reaction time of 6 h

the application of a molecular sieve had no significant effect on the synthesis, thus it was decided not to use a molecular sieve in the reaction for this study (data not shown). The synthesis was carried out for 6 h to ensure sufficient conversion.

Conversion rates of 81.35% and 98.81% were obtained using palmitic anhydride and vinyl palmitate, respectively (Fig. 8). When palmitic anhydride is used as an acyl donor, palmitic acid, a by-product generated through transesterification, can act as a reactant for esterification; therefore, puerarin palmitate can be synthesized at a high reaction rate. However, there is a limitation—a reverse reaction can be induced by water, a by-product of esterification. When vinyl palmitate is used as an acyl donor, vinyl alcohol is produced as a by-product after transesterification. As vinyl alcohol has an extremely unstable enol form, it is immediately converted to acetaldehyde through tautomerization; hence, the reverse reaction is not induced. Therefore, a higher conversion rate can be achieved by using vinyl palmitate than by using palmitic anhydride. For this reason, vinyl esters are preferred as acyl donors in acylation in many studies [51]. Since the ultimate goal of this study was the semi-continuous production of puerarin palmitate, preventing reverse reactions that could negatively affect the conversion rates in the long term was a priority. Therefore, vinyl palmitate, which does not induce the reverse reaction and can lead to a high conversion rate, was selected as the acyl donor for acylation.

Reaction time profile for the synthesis of puerarin palmitate

Next, we determined the optimal reaction time for synthesizing puerarin palmitate using TL GASG, with vinyl palmitate as the acyl donor. The reaction time was measured for up to 6 h at intervals of 0, 30, 60, 90, 120, 150, 180, 300, and 360 min; the conversion rates of the puerarin palmitate synthesis reaction using TL GASG were 0%, 66.34%, 82.48%, 89.54%, 93.36%, 95.55%, 97.17%, 98.46%, and 98.81%, respectively (Fig. 9). A conversion rate of 97.17% was achieved after 3 h, and no significant increase was observed thereafter. Guo et al. synthesized naringin palmitate via the esterification of naringin and palmitic acid, and the conversion rate was approximately 90% after 16 h [22]. Milivojević et al. synthesized phloridzin oleate, naringin oleate, and esculin oleate over 72 h, with conversion rates of 76.93%, 75.43%, and 78.11%, respectively [41]. These results showed that compared with the conversion rates achieved in previous studies on flavonoid ester synthesis, a high conversion rate was achieved in a short time in the present study (Table 1) [3, 4, 21, 26, 40, 41, 52, 53]. This underscores the successful development and application of the immobilized enzyme for synthesizing puerarin palmitate.

Reusability of immobilized enzyme for the synthesis of puerarin palmitate

The reusability of TL GASG as an immobilized enzyme for puerarin palmitate synthesis was investigated by repeatedly using the immobilized enzyme for 11 batch cycles and comparing the results with those of Lipozyme TL IM, a commercially available immobilized enzyme. Based on the reaction time profile results, the reaction time was set to 6 h. Both the immobilized enzymes maintained residual activities of more than 20% even after 11

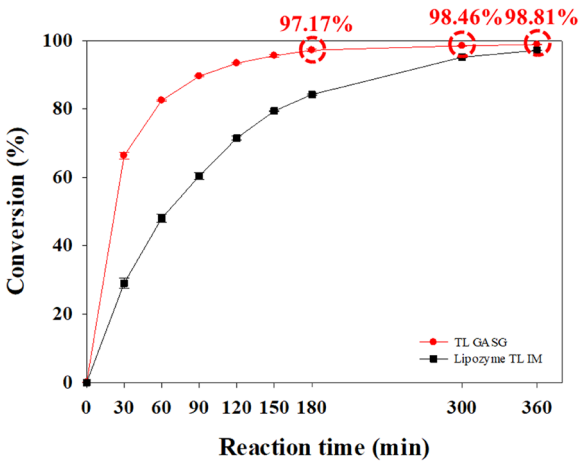


Fig. 9 Conversion of puerarin palmitate as a function of reaction time. Reaction conditions: 25 U/L of TL GASG and Lipozyme TL IM, 1:15 molar ratio of puerarin to vinyl palmitate, reaction temperature of 40 ℃, *tert*-amyl alcohol as the solvent, and a reaction time of 6 h

Table 1 Summary of reaction conditions for the enzymatic synthesis of flavonoid esters

Flavonoid	Acyl donor	Enzyme; enzyme concentration	Molar ratio (Flavonoid: Acyl donor)	Reaction temperature	Solvent	Reaction time; conversion rate	Refs.
Naringin	Oleic acid (C18)	Lipozyme TL IM; 10 g/L	1:20	40 °C	Acetonitrile	24 h; 92.17% 48 h; 93.10%	[3]
Naringin	Acetic anhydride, vinyl acetate (C2)	Lipozyme TL IM; 3 g/L	1:5	40 °C	Acetonitrile	8 h; 98.51%, 24 h; 97.54%	[4]
Naringin	Palmitic acid (C16)	Lipozyme TL IM; 50 g/L	1:10	50 °C	Acetone	16 h; ~ 90%	[26]
Naringin	Palmitic acid (C16)	Novozym 435; 10 g/L	1:5	60 °C	*tert*-Amyl alcohol	45 h; 43%	[40]
Neohesperidin	Vinyl esters (C2, C12, C16)	Liozyme TL IM; 43 g/L	1:8 (C12, C16) 1:12 (C2)	52 °C	*tert*-Amyl alcohol: DMSO = 4:1	24 h; 55% (C2), 83% (C12), 80% (C16)	[21]
Phloridzin, naringin, esculin	Oleic acid (C18)	CAL B immobilized onto a hydrophobic carrier; 2 g/L	1:18	65 °C	Acetonitrile	72 h; 76.93% (phloridzin), 75.43% (naringin), 78.11% (esculin)	[41]
Puerarin	Vinyl esters (C3-C8)	Whole-cell (*Aspergillus oryzae* GIM 3.4826); 50 g/L	1:30	40 °C	Tetrahydrofuran	24 h; 94.20% (C3), 95.90% (C4), 60.60% (C5), 93.40% (C6), 46.70% (C8)	[52]
Puerarin	Vinyl esters (C3, C6, C14)	Novozym 435; 2 g/L	1:30	50 °C	1-Ethyl-3-methylimidazoliumacetate	6 h; 63.91% (C3), ~ 40% (C6), ~ 35% (C14)	[53]
Puerarin	Vinyl palmitate (C16)	TL GASG; 27.9 g/L	1:15	40 °C	*tert*-Amyl alcohol	3 h; 97.17%	This study

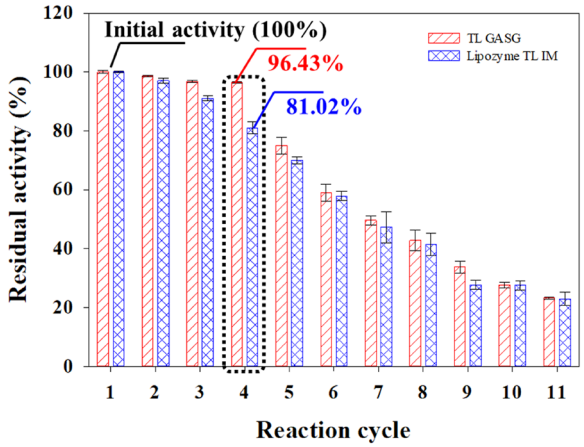


Fig. 10 Reusability of immobilized enzymes for the synthesis of puerarin palmitate. Reaction conditions: 25 U/L of TL GASG and Lipozyme TL IM, 1:15 molar ratio of puerarin to vinyl palmitate, reaction temperature of 40 ℃, *tert*-amyl alcohol as the solvent, and a reaction time of 6 h

cycles; TL GASG even showed more than 95% residual activity during the first four cycles (Fig. 10).

Gonçalves et al. used glutaraldehyde as a linker to immobilize porcine pancreatic lipase on activated carbon via covalent binding and used it to synthesize butyl butyrate. The conversion rate of this system was 66.26%, with a reusability of ≥ 86% (5 cycles) [45]. Yu et al. immobilized *Candida antarctica* lipase on MSU-H silica via covalent binding using glutaraldehyde as a linker. The conversion rate of ethyl esters of conjugated linoleic acid synthesis using this system was 46.9%, with a reusability of ≥ 90% (4 cycles) [35]. As the conversion rates obtained in the above-mentioned studies were lower than those achieved in the present study, it was confirmed that TL GASG had a higher conversion rate and reusability than those of other enzyme immobilization systems, including the commercially available immobilized enzyme Lipozyme TL IM (Table 2) [35, 41, 45, 46].

Table 2 Summary of the conditions for enzyme immobilization and the resulting enzyme reusability

Source	Support	Method	Product	Reaction time; conversion rate	Reusability	Refs.
Lipase from *Candida Antarctica*	ECR 8806 M (octadecyl functionalized hydrophobic resin)	Adsorption	Phloridzin oleate, naringin oleate, esculin oleate	72 h; 76.93%, 75.43%, 78.11%	≥ 70%, ≥ 60%, ≥ 40% (15 cycles)	[41]
Lipase from porcine pancreas	Activated carbon (from tamarind seeds)	Covalent binding (glutaraldehyde)	Butyl butyrate	4 h;66.26%	≥ 86% (5 cycles)	[45]
Lipase from *Thermomyces lanuginosus*	Immobead 150	Covalent binding (ethylenediamine)	Butyl butyrate, isoamyl butyrate	24 h; 60%	N/A	[35]
Lipase from *Candida antarctica*	MSU-H silica	Covalent binding (glutaraldehyde)	Ethyl ester of conjugated linoleic acid	32 h; 46.9%	≥ 90% (4 cycles)	[46]
Lipase from *Thermomyces lanuginosus*	Silica gel	Covalent binding (glutaraldehyde)	Puerarin palmitate	3 h; 97.17%	≥ 95% (4 cycles) ≥ 20% (11 cycles)	This study

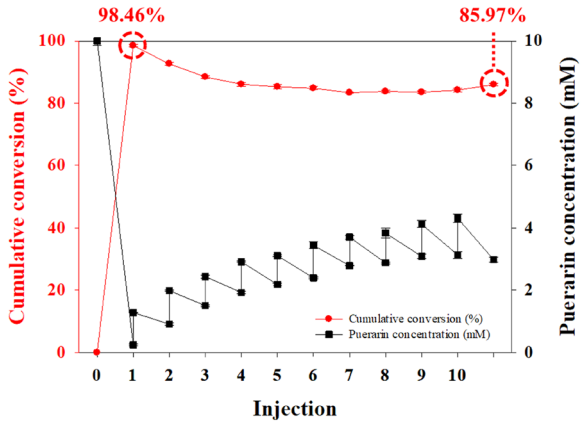


Fig. 11 Semi-continuous reaction for the synthesis of puerarin palmitate. Reaction conditions: 25 U/L of TL GASG, 1:15 molar ratio of puerarin to vinyl palmitate, reaction temperature of 40 ℃, and *tert*-amyl alcohol as the solvent

Semi-continuous reaction for the synthesis of puerarin palmitate

To determine the applicability of puerarin palmitate synthesis using TL GASG in a continuous reactor, a semi-continuous reaction was performed. Considering the results of the reaction time profile, the semi-continuous reaction started after the reaction had progressed sufficiently to reach a steady state. One milliliter was recovered at 30 min intervals, and 1 mL of substrate was newly supplied to maintain a constant reaction volume of 10 mL. The initial conversion rate before starting the semi-continuous reaction was 98.46%, and after semi-continuous recovery and supply, the cumulative conversion rate showed a gradual decrease, reaching a cumulative conversion of 84.77% after 5 injections. Maintaining a similar level, the cumulative conversion of 85.97% was maintained after 10 injections (Fig. 11).

El-Qelish et al. produced hydrogen semi-continuously by repeating the recovery of the reaction solution and supplying fresh substrate five times every 24 h for one week [54]. Similarly, Dineshkumar et al. semi-continuously produced lutein by repeating the recovery of the reaction solution and supplying a new substrate five times every 16 h [55]. Jiang et al. semi-continuously produced cyclohexanone through the hydrogenation of phenol in a ceramic membrane reactor using a Pd@CN catalyst. They maintained a 94.6% conversion rate of phenol and an 86.5% selectivity rate of cyclohexanone after 10 cycles [56]. In a similar case, although not semi-continuous, McNeff et al. reported that the conversion rate during biodiesel production through CSTR was maintained at an average of 87.5% [57]. Based on these results, it was determined that the semi-continuous production of puerarin palmitate performed in the present study maintained an appropriate cumulative conversion rate at a sufficient injection cycle.

Conclusion

In this study, TL lipase was immobilized on GASG to develop an immobilized enzyme with excellent stability, which was used for the synthesis of puerarin palmitate through a semi-continuous reaction. The developed immobilized enzyme, TL GASG, showed higher solvent and thermal stability than those of commercially used immobilized lipases. TL GASG demonstrated excellent operational stability, maintaining more than 20% residual activity during *p*-NPP hydrolysis and puerarin palmitate synthesis reactions even after 8 and 11 batch cycles, respectively. When TL GASG was used for the synthesis of puerarin palmitate, a conversion rate of 97.17% was achieved within 3 h. Furthermore, during the synthesis of puerarin palmitate in a semi-continuous system, a cumulative conversion rate of > 85% was achieved, even after 10 injections, confirming the applicability of TL GASG in a continuous reactor. Currently,

there are few studies on the synthesis of puerarin palmitate. Moreover, studies using the developed immobilized enzyme for flavonoid ester synthesis are scarce. This study is the first to demonstrate the application of an immobilized enzyme in puerarin ester synthesis and the use of a semi-continuous reaction for synthesizing flavonoid esters. The results of this study substantiate the feasibility of the continuous production of flavonoid esters and their diverse industrial applications, indicating that highly efficient continuous production of puerarin palmitate can be achieved via continuous reactor systems, optimization using the one-factor-at-a-time method, and response surface methodology for variable conditions. Furthermore, if the biomass extract from *P. lobata* is used as a feedstock for the continuous production of puerarin palmitate, it will also contribute to the development of a biorefinery strategy for the production of flavonoid esters, which we will explore in subsequent research.

Abbreviations

CAL B	*Candida antarctica* Lipase B
TL lipase	*Thermomyces lanuginosus* Lipase
RM lipase	*Rhizomucor miehei* Lipase
GASG	Glutaraldehyde-activated silica gel
TL GASG	*Thermomyces lanuginosus* Lipase immobilized on glutaraldehyde-activated silica gel

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Author contributions
HL: conceptualization, methodology, writing-original draft. EY: methodology, formal analysis, investigation. HKK: formal analysis, investigation, data curation. BKK: investigation, data curation. TL: validation, formal analysis, investigation. HYY: conceptualization, methodology, visualization, writing-review and editing. CP: conceptualization, validation, writing-review and editing, supervision, project administration, funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval and consent to participate
This manuscript is the original work of the authors and has not been previously published in another journal.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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