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Immobilization of Cellulase on a Reversibly Soluble−Insoluble Support: Properties and Application

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Cellulase was coupled to *N*-succinyl-chitosan (NSC) showing soluble−insoluble characteristics with pH change. Cellulase immobilized on NSC (NSCC) is in a soluble state during the enzyme reaction, yet can be recovered in its insoluble form by lowering the pH of the reaction solution after the reaction. NSCC was obtained under the optimized immobilization conditions of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) 10 mg, cellulase 15 mg, and pH 5.0. The retention activity of the immobilized cellulase was found to be 48.8%. The effects of pH and temperature on the activity and stability of NSCC were studied and compared with those of free cellulase. The optimum temperature and pH of NSCC was 45 °C and 4.0, respectively, which was found unchanged compared with the free one. The stability of cellulase against change in the pH and temperature was improved by the immobilization. The effectiveness of employing NSCC for extracting flavonoids from *Ginkgo biloba* leaf powder was investigated. Results showed that NSCC enhanced extraction yield up to 2.35-fold when compared with the conventional method. Moreover, NSCC retained 83.5% of its initial activity after five batches of hydrolysis reaction.

KEYWORDS: Cellulase; *N*-succinyl-chitosan; activity; stability; assisted extraction

INTRODUCTION

Cellulase can be used to disrupt the plant cell walls for assisting extraction of various kinds of compounds. Research in this area has been extensively reported and shown that enzyme-assisted extraction improves the yield of active compounds under mild conditions (*1*−*9*). However, these processes were apparently uneconomic because free cellulase was of low specific activity, susceptible to inactivation, and unable to recycle (*10*); meanwhile, polluting also occurred because the inactivated enzyme protein remained in the reaction solution. To solve these problems, common methods were to utilize enzyme immobilized on solid matrices (*10*, *11*) instead of the soluble form. Recovery and recycling of such immobilized enzyme can be done readily. However, in this case, there arises another problem, namely, that both cellulase bound on solid matrices and plant materials are not soluble, so cellulase will show poor performance due to diffusion limitations. Immobilizing the enzyme to reversibly soluble−insoluble supports has been suggested as a means to solve the problems inherent in the heterogeneous reaction systems. Enzyme immobilized on such supports could catalyze reaction in its soluble state and be water insoluble by simply adjusting the pH or temperature of the reaction solution upon recovery.

Several attempts have been reported previously for immobilization of cellulase on reversible soluble−insoluble polymers. Takeuchi and Makino (*12*) immobilized cellulase (cellulosin AC-8) on poly(L-glutamic acid) that was water-soluble in the neutral and alkaline solutions and insoluble by lowering the pH. Taniguchi et al. (*13*, *14*) covalently immobilized cellulase on an enteric coating polymer, Eudragit L, and (hydroxypropyl)methylcellulose acetate succinate (AS-L). Both enzyme supports were reversibly soluble and insoluble depending on the pH of the medium. This type of immobilized cellulase has been used to hydrolyze NaOH-treated cellulose, microcrystalline cellulose, and delignified rice straw. To our knowledge, cellulase immobilized on a reversibly soluble−insoluble support has not been utilized to hydrolyze plant material for extraction of active compounds.

In the present study, cellulase was immobilized on *N*-succinyl-chitosan (NSC). NSC is obtained by introducing succinyl groups into chitosan N-terminal of the glucosamine units. Its original use was for dressing materials, cosmetic materials, drug carrier, and wound dressings (*15*−*17*). NSC changes solubility with pH change. Then the immobilized cellulase (NSCC) was, for the first time, utilized to hydrolyze plant material for extraction of active compounds. *Ginkgo biloba* leaves were chosen as the model plant material because it is abound in nature and rich in flavonoid constituents. This is the first report regarding the application of reversibly soluble−insoluble immobilized cellulase on assisted extraction.

The purpose of this work was to (a) determine optimum conditions for immobilization of cellulase on NSC (the immobilization of cellulase to NSC employs the carbodiimide method with the condensing reagent EDC to couple carboxyl groups on NSC to the ε-amino groups of lysine residues in the protein); (b) determine the optimum pH, temperature, and stability of the immobilized enzyme in contrast to the free one; (c) evaluate the effectiveness of the immobilized cellulase on the extraction of flavonoids from *G. biloba* leaves in comparison to the conventional organic solvent method.

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MATERIALS AND METHODS

Materials. Cellulase from *Trichoderma viride*, chitosan (90% deacetylated chitin, MW 3.5×10^5), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), carboxymethylcellulose (CMC), succinic anhydride, dimethyl sulfoxide (DMSO), HPLC grade methanol, and other chemicals were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Quercetin (≥98%), kaempferol (≥98%), and isorhamnetin (≥98%) were obtained from Jiangxi Herbfine Hi-tech Co., Ltd. (Jiangxi, China).

G. biloba leaves, collected in Suzhou, were dried at 50 °C and then crushed to powder (particle size smaller than 300 μm).

Preparation of NSC. NSC was synthesized according to the method described in detail elsewhere (*18*). In brief, chitosan powder (2 g) was added to 40 mL of dimethyl sulfoxide (DMSO) with mixing. Then succinic anhydride was progressively added into the mixture, and the reaction occurred for a 6 h duration at 65 °C. The mixture was filtered to remove the solvent, and then the precipitate was dispersed in ethanol for 1 h at room temperature. The pH of the dispersion was adjusted by NaOH (1 M) to 10−12. The precipitate was collected by filtration and dispersed in 90 mL of distilled water. Then acetone (270 mL) was added, and the precipitate was washed with ethanol and acetone, respectively. Finally, the obtained product was dried at 50 °C under vacuum.

Immobilization of Cellulase (NSCC). NSC (0.5 g) was dissolved in 20 mL of citric acid/phosphate buffer solution (pH 5.0). EDC (10 mg) and cellulase (15 mg) were added to the above solution. After being stirred gently at room temperature for 4 h, the pH of the mixture was adjusted to 3.0 by HCl. The resulting precipitate was collected as the immobilized enzyme by filtration and then washed six times with 15 mL of citric acid/phosphate buffer solution (pH 3.0). Then the immobilized enzyme (NSCC) was dissolved in 20 mL of citric acid/phosphate buffer (pH 8.0) and stored at 4 °C. NSCC, prepared as previously described, was found to be soluble above pH 3.8 and essentially insoluble below pH 3.0.

Enzyme Activity Assays. The activity of cellulase was measured by using 1% (w/v) CMC as the substrate and acetate buffer (pH 4.0) as the medium. Free cellulase (1 mL) was added to 4 mL of the CMC solution and incubated at 45 °C for 25 min. The amount of generated glucose was determined by the 3,5-dinitrosalicylic acid agent (DNSA) method (*19*). One unit of activity was defined as 1 μmol of glucose/min.

The immobilized cellulase solution was added to the same assay medium and incubated at 45 °C for 25 min. Then the pH of the solution was adjusted to 3.0 by HCl. The resulting mixture was filtered, and the filtrate was collected to determine the amount of generated glucose.

The retained activity of immobilized cellulase, determined by the percentage of the activity of immobilized cellulase in the activity of free cellulase used for binding, was calculated according to the equation:

$$\text{retained activity}(\%) = \frac{U_{IC}}{U_{total}} \times 100 \quad (1)$$

where U_{IC} is the activity of immobilized cellulase and U_{total} is the activity of free cellulase used for binding.

Properties of the Free and Immobilized Cellulase. The activity assays were carried out over the pH range 3.0−8.0 and temperature range 35−65 °C to determine the pH and the temperature profiles for the free and immobilized cellulase. The results of pH and temperature are presented in a normalized form, with the highest value of each set being assigned the value of 100% activity.

The pH stability of the free and immobilized cellulase was ascertained by the residual activity of the enzyme exposed to 3.0−7.0 at 25 °C for 3 h. The thermal stability of free and immobilized cellulase was determined by the residual activity of enzyme exposed to various temperatures (35−55 °C) at pH 4.0 for 3 h. Activity of the samples was performed at optimum conditions.

Flavonoid Determination. The determination of flavonoids was carried out on a Shimadzu liquid chromatographic system (Shimadzu Co., Japan) consisting of LCsolution system software, LC-20AD pump, and Prominence SPD-M20A photodiode array detector (PAD).

The analytical column was a reverse-phase Shim-pack VP-DOS column (4.6mm × 250mm; Shimadzu, Japan). The mobile phase was methanol−0.4% phosphoric acid solution (55:45 v/v). The flow rate was 1 mL/min, the injection volume was 20 μL, the column temperature was maintained at 30 °C, and the retention times of reference compounds quercetin, kaempferol, and isorhamnetin were 12.097, 20.168, and 22.374 min, respectively. Reference compounds were monitored by a PAD at 360 nm.

Extraction of Flavonoids from *G. biloba* Leaves. *NSCC-Assisted Extraction*. Five grams of *G. biloba* leaf powder and the immobilized enzyme (buffer pH 4.0) were mixed together and incubated at 45 °C for a period of 3 h with a shaking speed of 150 rpm. After the enzyme treatment, *G. biloba* leaf powder was separated by vacuum filtration. The pH of the obtained filtrate was adjusted to 3.0 and the immobilized enzyme precipitated. The supernatant and 210 mL of ethanol were added into the enzymaticlly treated *G. biloba* leaf powder. The mixture was incubated at 70 °C for 2.5 h. After the extraction, the solid was again separated by vacuum filtration, and the supernatant was collected for further processing procedure as follows.

The obtained supernatant was shaken with petroleum ether (1:1 v/v) to extract the soluble pigment and repeated three times. The extraction raffinate was collected and subjected to solvent extraction again using ethyl acetate, repeated five times. The extracts were isolated and combined and then transferred to a rotary evaporator device (RE-52A; Shanghai Huxi Instrument, China) and evaporated to dryness under vacuum. Methanol (HPLC grade) was added to yield a solution. Then hydrolysis of flavonoids in the solution was performed as described by Hasler et al. (*20*). According to their results, flavonoid glycosides were reduced by hydrolysis to the three major aglycons (i.e., quercetin, kaempferol, and isorharmnetin) that were analyzed by HPLC. The obtained amount of aglycons could be correlated with the contents of total flavonoids. The average conversion factor was 2.51.

The extraction yield of flavonoids obtained was expressed as a percentage (w/w) as

$$\text{extraction yield}(\%) = \frac{W_{flavonoids}}{W_{leaf}} \times 100 \quad (2)$$

where $W_{flavonoids}$ is the weight of flavonoids extracted from *G. biloba* leaves and W_{leaf} is the weight of *G. biloba* leaf powder.

Free-Enzyme-Assisted Extraction. Five grams of *G. biloba* leaf powder and the free enzyme solution (buffer pH 4.0) were mixed together and incubated at 45 °C for a period of 3 h with a shaking speed of 150 rpm. After the enzyme treatment, 210 mL of ethanol was added. Then the mixture was immediately heated to 70 °C and incubated for 2.5 h. After extraction, the supernatant was collected for further processing procedure as described in NSCC-Assisted Extraction and then analyzed by HPLC to calculate the extraction yield of flavonoids.

Organic Extraction. Organic extraction was performed using a mixture of 30:70 (v/v) water/ethanol as the solvent at 70 °C for 2.5 h. In order to compare with the enzymatic extraction, 5 g of *G. biloba* leaf powder and 90 mL of buffer solution (pH 4.0) were first mixed together and incubated at 45 °C for a period of 3 h. Then 210 mL of ethanol was added, heated to 70 °C, and incubated for 2.5 h. After extraction, the supernatant was collected for further processing as described in NSCC-Assisted Extraction and then analyzed by HPLC to calculate the extraction yield of flavonoids.

Operational Stability. After each NSCC-assisted extraction run, the immobilized enzyme precipitated by adjusting the pH of the solution to 3.0 and was collected. Then the immobilized enzyme was suspended in a suitable volume of citric acid/phosphate buffer (pH 8.0) and was used to hydrolyze the next batch of *G. biloba* leaf powder as before to determine extraction yield again.

All of the experiments were carried out at least by triplicate, and the relative standard deviation was always under 5%.

RESULTS AND DISCUSSION

Optimum Conditions for Immobilizing Cellulase. To optimize the conditions of cellulase immobilization, the effects of the pH of the coupling buffer solution (3.0−8.0), EDC amount (5−50 mg), and cellulase amount (5−30 mg) on the activity of immobilized cellulase were investigated.

Effects of pH in Immobilization Procedure on the Activity of Immobilized Cellulase. In the immobilization procedure, EDC first reacts with the carboxyl groups on NSC, forming a reactive and unstable amine-reactive *O*-acylisourea intermediate.

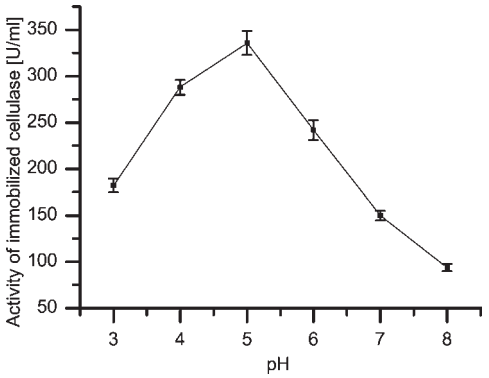

Figure 1. Effect of pH in immobilization procedure on the activity of immobilized cellulase. Immobilization conditions: binding time, 4 h; EDC amount, 30 mg; cellulase amount, 25 mg. Data represent the average ± standard deviation of three experiments.

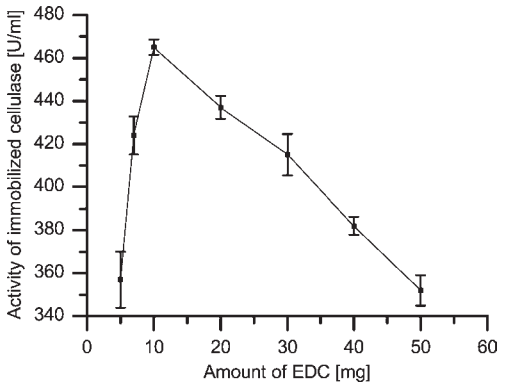

Figure 2. Effect of amount of EDC on the activity of immobilized cellulase. Immobilization conditions: binding time, 4 h; pH 5; amount of cellulase, 25 mg. Data represent the average ± standard deviation of three experiments.

This intermediate then reacts with the amine groups of enzyme, yielding stable amide bonds between enzyme and NSC.

The reaction was dependent on the pH value of the solution, so the effects of pH in immobilization procedure on enzyme activity were investigated first. Immobilization was carried out in buffer solution from pH 3.0 to pH 8.0. As shown in **Figure 1**, the maximum activity of immobilized cellulase reached 336 units/mL at pH 5.0. When pH was 3.0, NSC was insoluble and few carboxyl groups on NSC could be activated by EDC, so little enzyme protein binding was achieved and the activity of NSCC was low. When pH was above 4.0, NSC was soluble and the solution was homogeneous. At pH 5.0, EDC was in the most reactive state to react with carboxyl groups on the support, so the activity of NSCC increased when pH increased from 4.0 to 5.0. The significant decrease of NSCC activity above 6.0 was due to the instability of free cellulase against the increase of pH. pH 5.0 was accepted as the optimal pH condition for immobilization. Kang et al. (*21*) have also shown similar results, employing EDC as condensing reagent and coupling trypsin on poly(methyl methacrylate−ethyl acrylate−acrylic acid) latex particles, and found that a maximum value of enzyme activity was achieved at pH 5.0.

Effects of the Amount of EDC on the Activity of Immobilized Cellulase. The effects of the amount of EDC on immobilization were shown in **Figure 2**. An increase in the amount of EDC led to an increase in activity of the immobilized cellulase. The activity of the immobilized enzyme peaked when the amount of EDC was 10 mg and then decreased. At low amounts of EDC, it is probable that few bonds involving the support and the

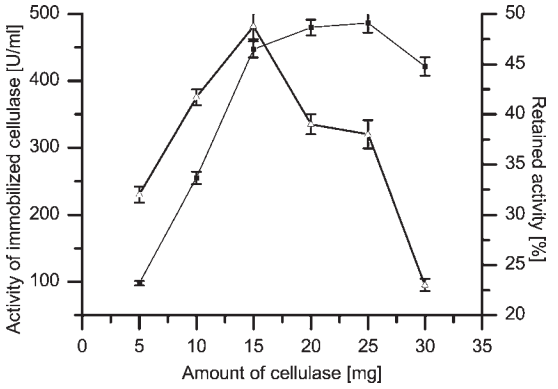

Figure 3. Effect of amount of cellulase on the activity of immobilized cellulase. (■) Activity of immobilized cellulase. (△) Retained activity. Immobilization conditions: binding time, 4 h; pH 5; EDC amount, 10 mg. Data represent the average ± standard deviation of three experiments.

enzyme molecules were formed and not sufficient to give a high immobilization yield. The increase in the amount of EDC resulted in more activated carboxyl groups and higher immobilized enzyme activity. However, when an excessive amount of EDC was introduced, the amide bonds can be formed not only between cellulase and support but also between enzyme molecules. This partial cross-linking of enzyme restricted the conformation mobility of the molecules and thus led to the loss in enzyme activity (*21*). Similar observations had already been reported by other researchers (*22*, *23*). In this study, the optimum EDC amount for cellulase immobilization was 10 mg.

Effects of the Amount of Cellulase on the Activity of Immobilized Cellulase. The amount of cellulase had an important effect on the activity and the retained activity of immobilized cellulase. It can be seen, from **Figure 3**, that with the increase of cellulase amount from 5 to 15 mg the retention of enzyme activity increased to a maximum value (48.8%). As the cellulase amount further increased from 15 to 30 mg, the retention of activity decreased rapidly to a minimum of 23%. Although, as the amount of cellulase reached 25 mg, the activity of the immobilized enzyme peaked, only slightly higher than that at the cellulase amount of 15 mg; however, the retention of activity decreased significantly. These results could be explained that at higher enzyme concentrations enzyme coupled to the support was oversaturated, which led to steric hindrance and resulted in lower activity yield (*21*). Thus the amount of cellulase (15 mg) was accepted as the optimal amount of enzyme for immobilization.

Properties of Immobilized Cellulase. *Effect of pH on Cellulase Activity*. The effect of pH on the activities of the free and immbolized cellulase was examined in the pH range 3.0−8.0. As shown in **Figure 4**, both free and immobilized enzymes were sensitive to the pH (optimal pH was 4.0). After immobilization, the pH response of the enzyme did not change. A similar observation for immobilized cellulase has been reported by Taniguchi et al. (*13*). They compared the activity of soluble and immobilized cellulase as a function of pH and found that both had the same optimum pH value of 4.8−5.1.

The pH stability of cellulase and immobilized cellulase was determined by measuring the residual activities of the enzyme kept in buffer solution with different pH 3.0−7.0 at 25 °C for 3 h before the activity measurement. As represented in **Table 1**, after immobilizing, the resistance of cellulase to pH was strengthened, and immobilized cellulase showed better pH stability than free enzyme following a 3 h incubation.

Effect of Temperature on Cellulase Activity. The optimum temperature curves for both free and immobilized cellulase were

shown in **Figure 5**. The temperature dependence of the enzyme activity was studied in the temperature range of 35−65 °C. As shown in **Figure 5**, maximum activity was observed at 45 °C for both free and immobilized enzymes. At low temperature, the free enzyme exhibited higher relative activity in comparison to the immobilized one. However, free cellulase was more sensitive to temperature increase, and the hydrolytic activity of free cellulase decreased sharply toward higher temperature. The immobilized cellulase was found to retain higher relative activities at higher temperature than the free one.

The optimum reaction temperature of the immobilized enzyme can be enhanced, diminished, or unchanged relative to free counterparts, and several examples of each kind have previously been reported (*24*, *25*).

Thermal stability of the free and immobilized cellulase was also investigated by incubation at 35−55 °C for 3 h before the measurements. As presented in **Table 2**, following heat treatment at 35, 40, 45, 50, and 55 °C for 3 h, the immobilized cellulase retained 102.0%, 102.0%, 100.0%, 98.0%, and 77.2% of its original activity, respectively, whereas free cellulase retained its activity at levels of 82.3%, 73.1%, 81.2%, 46.5%, and 36.5% during a 3 h incubation period, respectively. According to the above data, it can be seen that the immobilization of cellulase on NSC resulted in enhanced thermal stability, especially at higher temperature. At 55 °C, the activity loss was 23% for immobilized cellulase and 63% for the free one. As the carboxyl groups of NSC react with $-NH_2$ groups of the enzyme to form stable amide bonds, leading to the increase of the rigidity of enzyme structure (*26*), so immobilization resulted in better temperature stability.

Increase in thermal stability after immobilization has been widely reported in the case of cellulase and other enzymes (*25*, *27*−*31*). Dinçer et al. (*28*) observed that the cellulase immobilized on chitosan beads which are coated with maleic anhydride modified PVA membrane showed better activity at higher temperatures than the free enzyme after 1 h incubation.

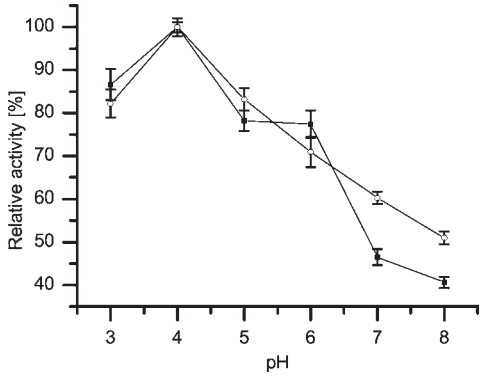

Figure 4. Effect of pH on the activities of free and immobilized cellulase. (■) Free cellulase. (○) Immobilized cellulase. Relative activity was calculated by using the highest activity of free and immobilized cellulase as 100%, respectively. Data represent the average ± standard deviation of three experiments.

Extraction of Flavonoids from *G. biloba* Leaves with Free and Immobilized Cellulase. Batches of *G. biloba* leaf powder were treated with free and immobilized cellulase, in different amounts, at 45 °C, pH 4.0. As the results showed in **Figure 6**, NSCC enhanced extraction yield when compared with the conventional organic extraction method (0.1448 ± 0.0040%) and increased the extraction yield as a function of the amount of enzyme. With the increase in NSCC amount, the extraction yield increased. When employing 15.36 units of NSCC for assisted extraction, the extraction yield was 0.3407 ± 0.0175%, which is 2.35-fold when compared with the conventional organic extraction method.

The effect of NSCC hydrolyzing *G. biloba* leaf powder was compared to that of the free cellulase. The results showed that the extraction yield of NSCC reached 91−97.5% of that of the free cellulase, however, notably higher than that of the conventional organic extraction, indicating that the immobilization of enzyme on NSC was effective in reducing mass transfer limitations.

In order to test the operational stability of NSCC, NSCC (15.36 units) was repeatedly used to hydrolyze five batches of *G. biloba* leaf powder at 45 °C, pH 4.0. The extraction yield decreased gradually in its successive cycles 1−5, dropping from 0.3407 ± 0.0175% to 0.3164 ± 0.0162%, 0.3137 ± 0.0120%, 0.3049 ± 0.0156%, and 0.2845 ± 0.0182%. The extraction yield in the fifth cycle dropped to 83.5% of its initial value. The result revealed that the activity of NSCC showed a decrease during the hydrolysis process, mainly due to the loss of enzyme with incomplete precipitation after each reaction run or by enzyme denaturation (*32*).

Plant cell walls, consisting of cellulose, hemicellulose, and pectin, are the barrier for the release of intracellular substances. So the extraction yield of flavonoids, employing the conventional organic extraction method, was low. Cellulase degraded the cell walls of *G. biloba* leaf and improved the release of flavonoids, so the extraction yield of flavonoids increased notably with cell walls degraded by free cellulase. However, free cellulase was uneconomic and tended

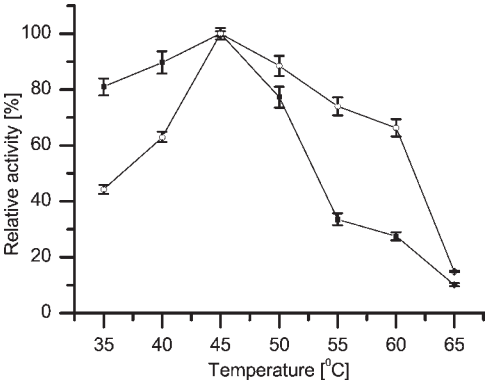

Figure 5. Effect of temperature on activities of free and immobilized cellulase. (■) Free cellulase. (○) Immobilized cellulase. Relative activity was calculated by using the highest activity of free and immobilized cellulase as 100%, respectively. Data represent the average ± standard deviation of three experiments.

Table 1. pH Stabilities of Cellulase[a]

	pH value				
	3.0	4.0	5.0	6.0	7.0
residual activity of immobilized cellulase (%)	102.0 ± 4.1	101.8 ± 3.8	119.7 ± 5.1	93.0 ± 3.0	77.0 ± 2.5
residual activity of free cellulase (%)	31.8 ± 1.4	75.2 ± 2.8	74.1 ± 0.8	30.5 ± 0.8	20.9 ± 0.9

[a] Residual activity was calculated by using the initial activity of free and immobilized cellulase as 100%, respectively. Data represent the average ± standard deviation of three experiments.

Table 2. Thermostabilities of Cellulase[a]

	temperature (°C)				
	35	40	45	50	55
residual activity of immobilized cellulase (%)	102.0 ± 1.4	102.0 ± 2.0	100.0 ± 1.9	98.0 ± 2.8	77.2 ± 3.6
residual activity of free cellulase (%)	82.3 ± 4.0	73.1 ± 2.0	81.2 ± 3.2	46.5 ± 1.6	36.5 ± 1.8

[a] Residual activity was calculated by using the initial activity of free and immobilized cellulase as 100%, respectively. Data represent the average ± standard deviation of three experiments.

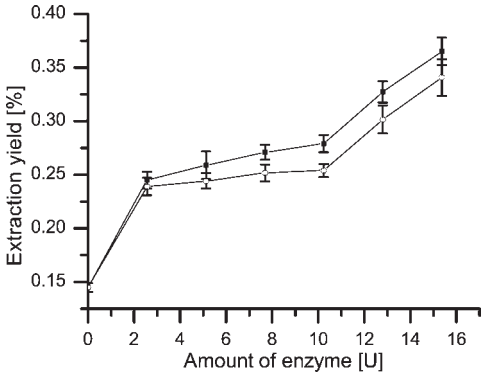

Figure 6. Effect of free and immobilized cellulase on extracting flavonoids from *G. biloba* leaves. (■) Free cellulase. (○) Immobilized cellulase. Data represent the average ± standard deviation of three experiments.

to pollute the extraction solution because, at the end of reaction, it had to be inactivated, and the inactivated enzyme protein remained in the solution.

NSCC proved to be effective in the assisted extraction of flavonoids from *G. biloba* leaf powder with reasonable extraction yield compared to the free cellulase extraction. The reason was that NSCC was completely soluble and thus ensured full contact between the substrate and enzyme. Moreover, NSCC could be reused by simply adjusting the pH value of the reaction medium, hence avoiding polluting the extraction solution and decreasing the cost. In conclusion, NSCC could not only keep the yield of the free-enzyme-assisted extraction but also realize the reuse of enzyme. Therefore, the proposed method of immobilizing cellulase on a reversibly soluble−insoluble support provides an effective potential approach in assisted extraction of active compounds from plant materials.

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