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Aqueous Solubility and Stability Enhancement of Astilbin through Complexation with Cyclodextrins

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ABSTRACT: The complexation of astilbin with α-, β-, and γ-cyclodextrin (CD) was studied by phase solubility test and UV−vis spectral titration. Complexation with CDs gradually decreased the absorbance of astilbin at 291 nm and obviously increased its water solubility. The formation constant (K_a) between astilbin and the three CDs was calculated. The stability of astilbin complexes increased in the order α-CD < γ-CD < β-CD, attributed to the CDs' cavity size. Temperature studies showed that the K_a value decreased along with the rise of temperature. The negative values of enthalpy and entropy during complexation indicated that the complexation process was enthalpy-controlled. In alkaline medium isomerization and decomposition of astilbin were found; however, the addition of CDs significantly improved its stability through complexation. The solubility of astilbin in β-CD microcapsules prepared by the freeze-drying method was enhanced by 122.1-fold, and its dissolution profile was improved.

KEYWORDS: *astilbin, cyclodextrin, complexation, solubility, stability*

INTRODUCTION

Flavonoids are polyphenolic compounds present in many fruits, vegetables, and herbs.[1] These compounds are proposed to have health-promoting effects by reducing the risk of cardiovascular disease and neurodegenerative disorders, as well as cancer.[2−4] However, the poor solubility and instability of these compounds may constitute a serious problem in their bioavailability. Cyclodextrins (CDs) are a group of cyclic oligosaccharides resulting from the degradation of starch by cyclomaltodextrin glucanotransferase. Due to their special structure of hydrophobic inner cavity and hydrophilic outer surface, CDs form inclusion complexes with various guest molecules through noncovalent interactions.[5] The complexation improves the solubility, stability, and bioactivity (e.g., antioxidation) of guest molecules,[6−13] which has many applications in the pharmaceutical, cosmetics, and food industries.[14,15] CDs can also be used as a functional drug carrier to enhance the drug absorption across biological barriers and control the rate and/or time profile of drug release.[16]

Astilbin, (2*R*,3*R*)-taxifolin-3-*O*-*R*-L-rhamnopyranoside, is a dihydroflavonol that is widely distributed in many plants, such as *Engelhardia roxburghiana*,[17] *Hypericum perforatum*,[18] *Smilax* genera plants,[19] and grape.[20] It is the dominant compound of *Rhizoma Smilacis Glabrae* and of processed functional food, turtle jelly.[21,22] The content of astilbin in 21 commercial wines from France is in the range of 0.77−15.12 mg/L, with an average level of 5.21 mg/L.[20]

The most interesting pharmacological potential of astilbin is its novel immunosuppressive activity. Xu et al. revealed that astilbin could selectively inhibit the effector but not the induction phase of delayed-type hypersensitivity without effect on humoral immunity; it selectively facilitates the apoptosis of activated T cells but has no effect on naive T cells. These characteristics are distinct from the commonly used immunosuppressant cyclosporin A, which usually results in general suppression of the whole immune system with various side effects.[23−26] Besides, astilbin has also showed antioxidative,[27] antibacterial,[28] and 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibition activities.[29] Unlike other flavonoid glycosides that are usually absorbed after hydrolysis, astilbin can be absorbed in its intact form and as the main metabolite of 3′-*O*-methylastilbin. However, the oral absorption of astilbin in vivo was very poor, with an absolute bioavailability of 0.066% in rat,[30] possibly due to its poor solubility. By the development of an astilbin self-microemulsifying drug delivery system, its oral bioavailability was 5.59-fold enhanced in beagle dogs.[31]

In the present study, the three most common native CDs, α-, β-, and γ-CD, were used for the encapsulation of astilbin to improve its solubility and stability. The complexation reaction was studied in detail, and various thermodynamic parameters such as formation constant (K_a), enthalpy (ΔH), and entropy (ΔS) were calculated. The protection effect of CDs for astilbin in alkaline medium due to complexation was studied and discussed. Furthermore, the solubility profile of microcapsules of astilbin with the three CDs prepared by a freeze-drying method was studied.

MATERIALS AND METHODS

Chemicals. Astilbin (>98%) was purified from *Rhizoma Smilacis Glabrae* by preparative HPLC in our laboratory and was identified by UV, IR, MS, and NMR. α-, β-, and γ-CD were purchased from Jiangsu Fengyuan Biotechnology Co. Ltd. (Jiangsu, China) and were used without further purification. HPLC grade acetonitrile was purchased from RCI Labscan LTD (Bangkok, Thailand). Double-distilled water was used throughout the study. All other reagents and chemicals employed were of analytical reagent grade.

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UV−Vis Spectral Titration. The absorbance measures were carried out on an Analytik Jena-Specord 200 spectrophotometer (Germany) with matched 10 mm quartz cells. A 1 mL aliquot of the stock solution of astilbin (0.6 mM) prepared with 50% methanol was transferred into a 10 mL volumetric flask, and then an appropriate amount of CD solution (10 mM in water) was added. The mixture was diluted to the mark with double-distilled water or acetate buffer (pH 3.5, 0.2 M). The concentration of astilbin was held constant at 0.06 mM, whereas the final concentrations of CDs varied from 0 to 1.4 mM. After thorough shaking, the mixture was allowed to stand for 15 min at room temperature (~298 K). The UV−vis spectra were measured against a corresponding reagent blank without astilbin. All experiments were carried out in triplicate.

Phase Solubility Studies. Phase solubility studies were performed according to the method described by Shehatta with some modifications.[32] An excess amount of astilbin (10 mg) was added to 3 mL of water containing various concentrations of the studied CDs (ranging from 0 to 5 mM). The mixture was shaken at a speed of 120 rpm for 7 days under different temperatures (293, 298, 303, 308, and 313 K). Saker incubator SKY-2112B produced by SUKUN (Shanghai, China) was used for the study. After equilibrium was attained, undissolved astilbin was removed by filtration through 0.45 μm filters. The concentration of astilbin in the clear filtrate was appropriately diluted and determined spectrophotometrically at 291 nm (ε = 1.41 × 10^4 M^{-1} cm^{-1}). The studies were carried out in triplicate at each temperature.

Aqueous Stability Tests. The stability of astilbin in acidic (0.2 M acetate buffer, pH 3.5) and alkaline solution (0.2 M phosphate buffer, pH 8.0) was studied at room temperature (~298 K), respectively. A 1 mL aliquot of the astilbin solution (1 mg/mL in 50% methanol) was mixed with 5 mL of buffer solution containing no or 6 mM CDs. At different time intervals, the remaining astilbin was determined by isocratic HPLC with injection volume of 10 μL. A mobile phase of 25% acetonitrile was used. Monitoring was performed at 291 nm, and the column temperature was set at 313 K. HPLC analyses were performed on a Waters 600 HPLC system equipped with a dual wavelength detector (waters 2487). An Agilent Zorbax SB C18 column (250 mm × 4.6 mm i.d., 5 μm) was used.

HPLC-MS/MS analyses were performed on an Agilent 1100 system (Palo Alto, CA, USA) together with an AB MDS Sciex (Concord, ON, Canada) API 2000 triple-quadrupole mass spectrometer equipped with a Turbo IonSpray ESI source. The experimental conditions (column, flow rate, mobile phase, injection volume) were the same as described above. Only about 35% of eluent was introduced into the ESI source after split. The mass spectrometer was operated in the negative ion mode. Ultrapure nitrogen was used as nebulizer, curtain, and collision-activated dissociation gas at 20, 10, and 1 (instrument units), respectively. The optimized Turbo IonSpray voltage and temperature were set at −4500 V and 673 K, respectively.

Preparation of Molecular Microcapsules. A fourth of a gram of astilbin dissolved in 1 mL of methanol was mixed with an equimolar amount of CD dissolved in 25 mL of distilled water. The mixture was shaken at 5 h at a speed of 160 rpm. Then, the solution was freeze-dried, and the solid microcapsules were stored in a desiccator. A vacuum freeze-dryer (FD-1-50, Boyikang Experimental Apparatus Co. Ltd., Beijing, China) was used.

Dissolution Studies. The dissolution rates of astilbin and its CD microcapsules were measured by using the dispersed amount method. The powder sample (containing 25 mg of astilbin) was added to 100 mL of water and stirred at 100 rpm at 310 K. At appropriate intervals, an aliquot (0.5 mL) of the dissolution medium was withdrawn using a pipet with a cotton plug. The volume in the vessel was replaced with water after each sampling. After filtration through 0.45 μm filters, the astilbin concentrations in sample solutions were determined spectrophotometrically at 291 nm.

Statistical Analysis. Data were expressed as the mean ± standard deviation (SD) of triplicates. Statistical calculations were carried out using OriginPro version 6.0 software (OriginLab Corp., Northampton, MA, USA). *P* values of <0.05 were regarded as significant, and *P* values <0.01 were regarded as very significant.

RESULTS AND DISCUSSION

UV−Vis Spectral Characterization. Flavonoids are a well-defined group of compounds with very characteristic UV−vis spectra. As shown in Figure 1A, the UV−vis spectra of astilbin

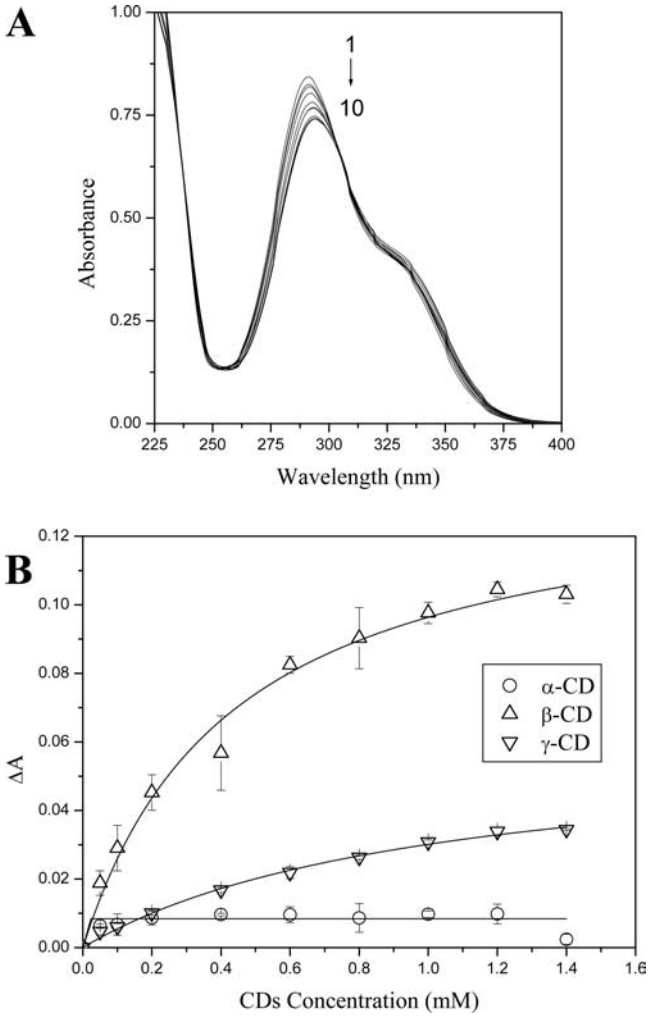

Figure 1. (A) UV−vis spectral changes in astilbin (0.06 mM) upon addition of β-CD (0−1.4 mM, from 1 to 10) in double-distilled water; (B) absorbance change (ΔA) of astilbin at 291 nm as a function of different CD concentrations in acetate buffer (0.2 M, pH 3.5); the lines show the best fits to eq 1.

presented maximum absorption at 291 nm and a shoulder peak at 325 nm, which is in accordance with the characteristic absorbance of dihydroflavonol that exhibits two maximum absorbance bands: band I (300−400 nm) and band II (270−295 nm). Its UV−vis spectra were the same in the pH range of 2.0−6.0. However, when the medium became basic, its maximum absorption peak shifted to 325 nm. The phenomenon may be due to the dissociation of hydroxyl groups in the molecules. As reported by Teixeira et al.,[33] the pK_a values of taxifolin (the aglycon of astilbin) corresponding to mono-, di-, and triprotonation were 6.68, 8.89, and 10.95, respectively.

Inclusion complex formation with CDs often causes UV−vis spectral changes of substrates.[34] Figure 1A shows the UV−vis spectra of astilbin in the absence and presence of β-CD. With the addition of β-CD, the absorption maximum of astilbin at 291 nm red-shifted to 296 nm, whereas the absorbance decreased gradually. An isosbestic point also appeared at 305

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Table 1. Stability Constant (K_a, M^{-1}) for the inclusion Complexation of Astilbin with CDs in Double-Distilled Water and Acetate Buffer (0.2 M, pH 3.5) Calculated by UV−Vis Spectral Titration Method

	acetate buffer			double-distilled water		
CD	K_a	ΔA_{max}	R^2	K_a	ΔA_{max}	R^2
α-CD	–[a]	–	–	–	–	–
β-CD	2305.9 ± 346.2	0.138	0.989	1788.5 ± 273.4	0.144	0.991
γ-CD	995.2 ± 123.8	0.062	0.996	482.6 ± 118.9	0.086	0.992

[a]Unable to calculate.

nm. These phenomena suggested the formation of astilbin−β-CD complexes. These changes might be attributed to the shielding of chromophore groups by CD cavity due to complex formation. Similar spectral changes were also observed upon addition of γ-CD to astilbin solution. However, α-CD did not show any effect.

To evaluate the formation constant (K_a) between astilbin and CDs from these spectral changes, the decreases in absorbance at 291 nm with increasing concentrations of CDs were measured. Assuming that the astilbin and CDs formed 1:1 complexes, the complexation reaction could be expressed as

$$\text{astilbin} + \text{CD} \overset{K_a}{\leftrightarrow} \text{astilbin} - \text{CD}$$

The measured absorbance of astilbin can be related to the total CD concentration by eq 1.[35]

$$A = A_0 + \frac{\Delta A_{max} K_a [\text{CD}]}{1 + K_a [\text{CD}]} \quad (1)$$

where A and A_0 are the absorbance of astilbin at 291 nm in the presence and absence of CD, respectively. [CD] is the concentration of CD. The K_a value could be calculated through nonlinear fitting of eq 1 to the experimental data.

Figure 1B illustrates the curve-fitting plots for the titration of astilbin with the three CDs. The excellent fit between the experimental and calculated data (all correlation coefficients >0.989, except for α-CD) indicated that the stoichiometry of astilbin with β- and γ-CD was 1:1. The calculated K_a values are listed in Table 1. As shown, the K_a values of astilbin with β- and γ-CD were 2305.9 ± 346.2 and 995.2 ± 123.8 in acetate buffer at pH 3.5, respectively. However, in distilled water medium (pH 6.6), the K_a values decreased. The results suggested that the interaction of CDs with ionized astilbin was weaker than that of un-ionized species. Similar results have been observed in many other CD/guest complexation studies.[36] It was also noted that the stability of astilbin inclusion complexes was in the order β-CD > γ-CD > α-CD. These differences were due to the variety of cavity size of the three CDs. The cavity diameters of α-, β-, and γ-CD are 0.49, 0.62, and 0.79 nm, respectively.[5] During the complexation of CDs with guest molecules, the molecule fitness notably affected the van der Waals force, which is a major driving force in the complexation process. Because of cavity size, α-CD interacts well with aliphatic chains, whereas β-CD is appropriate for aromatic rings. The results indicated that the cavity size of β-CD permitted stronger interactions with astilbin, whereas the cavities of α- and γ-CD were too small and too large, respectively, thus reducing their affinity with astilbin.

Solubility Studies. Astilbin was insoluble in chloroform and petroleum ether and had good solubility in methanol and ethanol. Its solubility in water at different temperatures is shown in Figure 2A. As shown, the solubility of astilbin at 298 K was only 0.491 mM (0.221 g/L). According to the Chinese

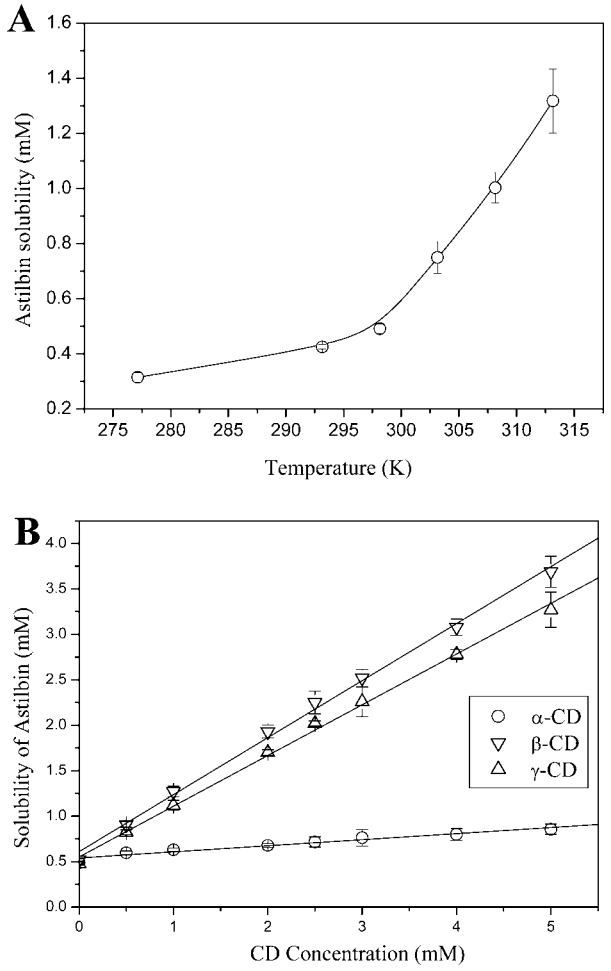

Figure 2. (A) Solubility of astilbin at different temperatures; (B) solubility phase diagrams of astilbin in water with different concentrations of CDs at 298 K.

Pharmacopoeia, it is a poorly soluble compound.[37] With the rise of temperature, its solubility quickly increased.

Figure 2B shows the phase solubility diagrams for astilbin with the three CDs at 298 K. As shown, the concentration of dissolved astilbin increased constantly with the rise of CD concentration. The solubility enhancement effect increased in the order α-CD < γ-CD < β-CD. Because the cavity of α-CD was too small for astilbin to enter, its effect was very weak. However, the solubility enhancement effect of β-CD and γ-CD was notable. With 5 mM β-CD and γ-CD, the solubility of astilbin was increased 7.58 ± 0.35 and 6.71 ± 0.39 times, respectively. The phase solubility diagrams were all of the A_L type following the Higuchi and Connors classification, indicating the formation of 1:1 complexes in all of the CDs studied.[32] K_a values were calculated from the straight-line

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portion of the phase solubility diagram according to the Higuchi–Connors equation

$$K_a = \frac{\text{slope}}{S_0(1 - \text{slope})} \quad (2)$$

where S_0 is the solubility of astilbin in the absence of CD. The calculated K_a values are listed in Table 2. As shown, the K_a values obtained by using the physical method were similar to those obtained with the spectral titration method (Table 1), indicating the validity of both methods.

Table 2. K_a Values (M^{-1}) and Other Thermodynamic Parameters for the Inclusion Complexation of CDs with Astilbin in Double-Distilled Water at Different Temperatures Calculated by Phase Solubility Test

	α-CD	β-CD	γ-CD
293 K	127.9 ± 5.7	2260.6 ± 57.4	1674.7 ± 41.8
298 K	138.2 ± 13.4	2287.1 ± 49.4	1689.3 ± 40.0
303 K	66.1 ± 8.6	1757.1 ± 30.8	1327.6 ± 24.3
308 K	46.9 ± 3.8	1305.3 ± 30.8	957.7 ± 19.3
313 K	148.2 ± 16.1	812.9 ± 34.6	772.7 ± 36.8
$-\Delta G$ (kJ/mol, 298 K)	11.9	18.8	18.01
$-\Delta H$ (kJ/mol)	82.7	52.7	41.5
$-\Delta S$ (J/K·mol)	237.1	111.9	77.3

Effect of Temperature on Complexation. The effects of temperature on the complexation of astilbin with CDs were studied by phase solubility tests at 293, 298, 303, 308, and 313 K, respectively. Using eq 2 as described above, the K_a values at different temperatures were calculated. These values were further used for calculating the enthalpy and entropy parameters through the equation $\Delta G = -RT \ln K_a = \Delta H - T\Delta S$. ΔH and ΔS values were derived from the linear regression of $\ln K_a$ versus $1/T$.

As shown in Table 2, for all CDs, the K_a values decreased with the rise of temperature, indicating that the affinity between CDs and astilbin decreased. Lower temperature facilitated the complexation process. The negative value of ΔG suggested that the complexation was a spontaneous process and thermodynamically favored. Negative ΔH and ΔS values implied that the complexation process was exothermic and entropically unfavorable. In theory, negative enthalpy arose from the van der Waals interaction and the displacement of high-enthalpy water molecules from CDs cavity. The negative entropy change is caused by the steric barrier of the CD cavity to the freedom of shift and the rotation of the guest molecule.[5]

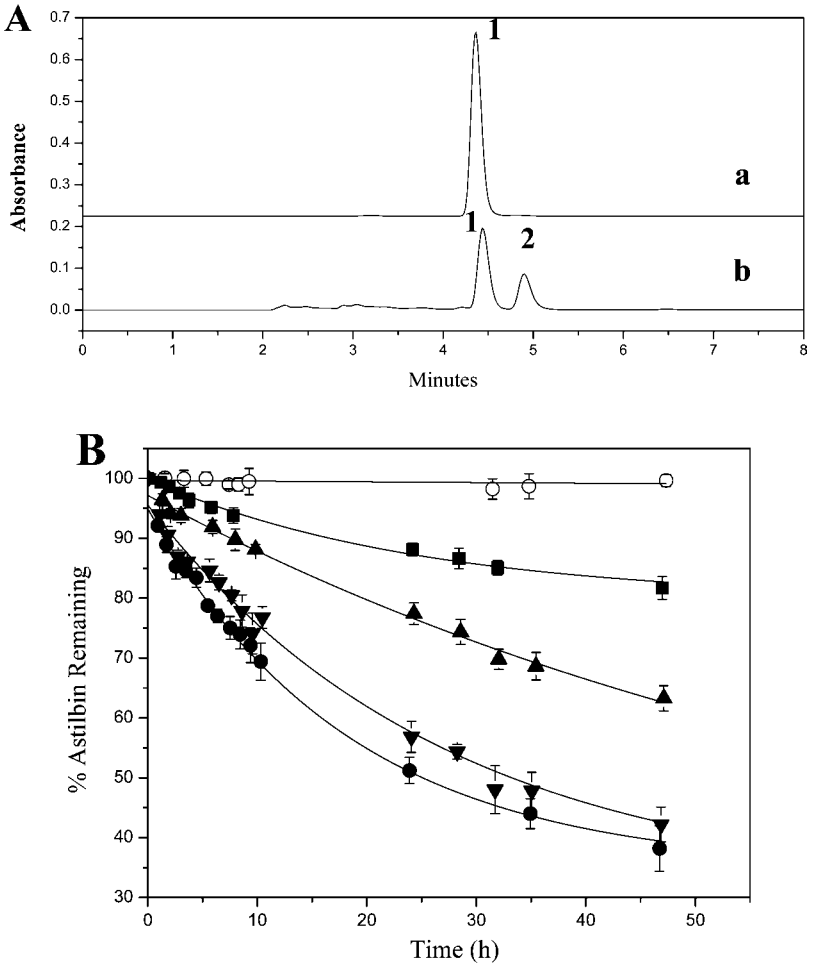

Figure 3. (A) HPLC chromatogram of astilbin in pH 8.0 phosphate buffer at 0 h (a) and at 36 h (b): peak 1, astilbin. (B) Time course for loss of astilbin in different aqueous solutions: (○) acetate buffer at pH 3.5; (●) phosphate buffer at pH 8.0; (○) phosphate buffer at pH 8.0 with 5 mM of α-CD; (■) phosphate buffer at pH 8.0 with 5 mM of β-CD; (▲) phosphate buffer at pH 8.0 with 5 mM of γ-CD. Results are shown as percent astilbin remaining as determined by HPLC peak area.

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Aqueous Stability of Astilbin. The aqueous stability of astilbin in acidic and basic media was studied. The results showed that astilbin was stable in acidic solution (pH 3.5). However, it was unstable in alkaline solution. As shown in Figure 3A, a new peak (peak 2) appeared in the HPLC chromatogram of astilbin after storage in pH 8.0 phosphate buffer. HPLC-MS/MS analysis showed that peak 2 had a molecular ion at m/z 449 $[M - H]^-$ and characteristic ions at m/z 303, 285, and 151, which were exactly the same as those of astilbin. Astilbin is a dihydroflavonol with two chiral carbon atoms in positions 2 and 3 and, therefore, has four enantiomeric forms.[38] Thus, peak 2 was identified as one enantiomer of astilbin. Gaffield et al. have studied the isomerization of astilbin in many media and conditions.[39] However, the isomerization of astilbin in alkaline medium was found for the first time.

Figure 3B shows the time course results for the loss of astilbin under different aqueous conditions. As shown, the content of astilbin was almost unchanged at pH 3.5 during 48 h. However, its content quickly decreased over time in the weak alkaline solution (pH 8.0). Our present study revealed that alkaline medium could accelerate the isomerization of astilbin. However, the area sum of peaks 1 and 2 gradually decreased with time, which meant that besides isomerization, the decomposition of not only astilbin but also its enantiomer occurred in alkaline solution.

With the addition of CDs in the alkaline solution, the rate of decrease of astilbin slowed. After 48 h, the remaining astilbin in phosphate buffer at pH 8.0 with 0 or 5 mM α-, γ-, and β-CD was 38.1 42.2, 63.2, and 81.7%, respectively. β-CD showed the best protection effect, and α-CD was the worst. The results were in accordance with the thermodynamic study that the stability of the complexes were in the order β-CD > γ-CD > α-CD. The stability improvement of astilbin was due to the protection effect of the CD cavity after complexation. Miyake et al. also reported that the addition of CD could reduce the hydrolysis rate of rutin in alkaline solution.[40]

Characterization of Astilbin–CD Microcapsules. As revealed by phase solubility test and UV–vis spectral titration, astilbin and CDs form 1:1 complexes in the solution, and their microcapsules were prepared by a freeze-drying method at a molar ratio of 1:1. The solubility of astilbin in the different microcapsules was determined. As shown in Figure 4A, the solubility of astilbin in all CD microcapsules was improved. Most notably, its solubility in β-CD microcapsules was 0.060 M at 298 K, which was 122.1 times increased compared to astilbin alone. Figure 4B shows the dissolution profiles of astilbin and its CD complexes in water at 310 K. It was apparent that the complexes dissolved much more rapidly than astilbin alone. The enhanced dissolution rate of astilbin may be due to the increase in solubility and wettability by inclusion complexation.

Although astilbin exhibits many interesting bioactivities, the properties of poor solubility and instability may limit its applications. From the results reported in this study, attributed to the cavity size, β-CD could form the most stable complexes with astilbin among the three native CDs. The complexation could significantly improve the stability of astilbin in alkaline medium. The solubility of astilbin in β-CD microcapsules was 122.1 times increased, and its dissolution profiles were improved. The results suggested that β-CD has a significant advantage in the development of novel astilbin formulation for functional beverages or healthcare products.

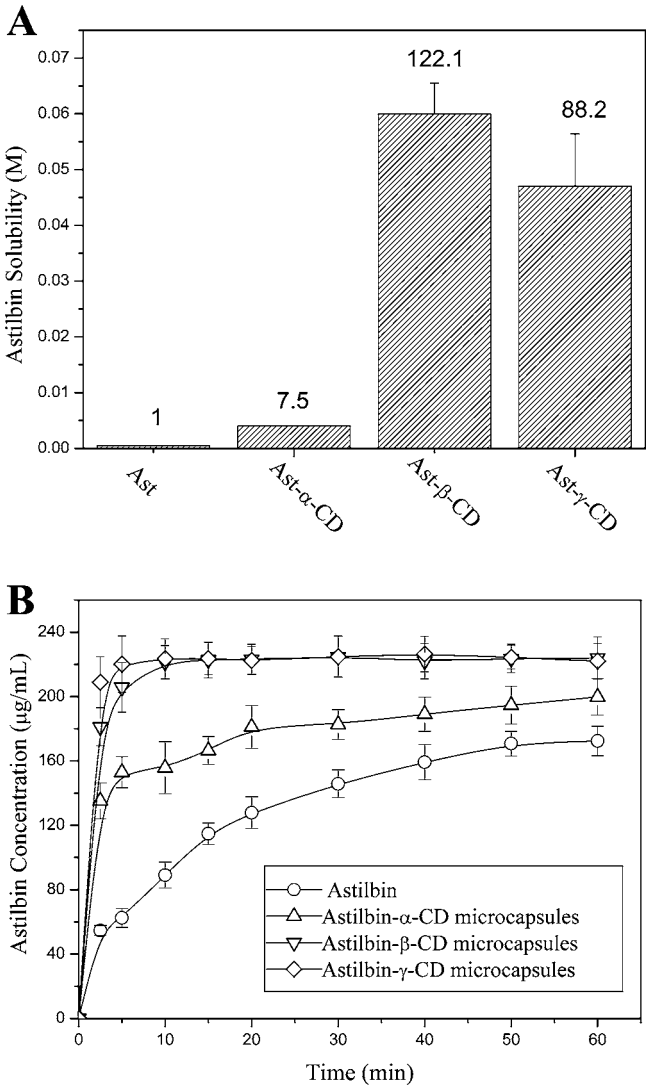

Figure 4. (A) Solubility of astilbin in different CD microcapsules at 298 K; (B) dissolution profiles of astilbin and its CD microcapsules in water at 310 K. The microcapsules of astilbin and CDs were prepared by a freeze-drying method at a molar ratio of 1:1.

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Notes

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