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Macroporous Silica Microcapsules Immobilizing Esterase with High Hydrolysis Reactivity

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Abstract

An esterase, 3,4-dihydrocoumarin hydrolase, was directly immobilized into silica microcapsules. The hydrolysis reaction of 3,4-dihydrocoumarin by a macroporous silica microcapsule immobilizing the esterase was faster than those by mesoporous ones. Using this macroporous microcapsule, the hydrolysis reaction of *p*-nitrophenyl acetate proceeded with comparable rate to non-immobilized esterase.

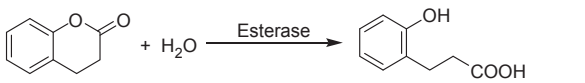
Keywords: Macroporous | Silica microcapsule | Enzyme immobilization

Immobilization of enzymes is an essential technology for their practical and industrial applications.[1–4] Since amorphous silicas are generally safe materials (for example; used as food additives) and practically insoluble in common water and organic solvents, they are well used as the supports of immobilized enzymes.[5–8] However, their small pore voids often restrict the access of reactants to the immobilized enzymes to slow reaction rates. We have studied the preparations of amorphous silica microcapsules using W/O/W (water/oil/water) emulsion with sodium silicate solution.[9–14] When enzymes are added to the solution, the enzymes are directly immobilized into the silica microcapsules.[12–14] Hyperthermophilic β-glucosidase immobilized into the microcapsule is effective for the hydrolysis reaction of cellobiose.[14] However, the reaction rate is significantly slower than that of non-immobilized one, because the narrow mesopore of the silica microcapsule (about 2.6 nm) suppresses the access of cellobiose to β-glucosidase. Therefore, the optimization of the pore properties of the microcapsule is required. On the other hand, we also found methods for producing silica microcapsules bearing macropores by the addition of water-soluble polymers to a sodium silicate solution.[10,11] It is expected that macroporous silica microcapsule encapsulating enzyme facilitates the access of reactant to enhance the reaction rate. Here we focus on the preparation of silica microcapsules immobilizing an esterase, 3,4-dihydrocoumarin hydrolase [EC 3.1.1.35] obtained from *Acinetobacter calcoaceticus* F46, because this esterase is reported to catalyze the hydrolysis of 3,4-dihydrocoumarin very rapidly,[15] being beneficial for evaluating the effects of the pore properties of silica microcapsules on the reaction rate of the enzyme. In this article, we report the behaviors of the hydrolysis reactions of carboxylic acid esters catalyzed by the esterase immobilized into silica microcapsules toward developing our microcapsule technology.

Table 1. Profiles of silica microcapsules

Capsule No.	Precipitant	Enzyme content[a] (wt%)	SSA[b] (m^2/g)	PV[c] (mL/g)	PPD[d] (nm)
Cap0	NH_4Cl	—	449	0.343	3.71
Cap1	NH_4Cl	18.7	273	0.258	3.28
Cap2	$(NH_4)_2SO_4$	14.4	377	0.413	4.19
Cap3	NH_4HCO_3	15.7	293	0.412	4.19
Cap4	$KHCO_3$	11.3	263	0.536	9.23
Cap5[e]	$KHCO_3$	11.4	333	0.362	4.19
Cap6[f]	$KHCO_3$	15.7	292	0.381	4.76
Cap7[g]	$KHCO_3$	2.5	446	0.419	4.76

[a]Estimated from the weight decrease from 200 to 500 °C in TG-DTA analyses. [b]Specific surface area calculated by BET plot. [c]Pore volume of mesopore estimated by BJH method using adsorption branches. [d]Peak pore diameter of mesopore estimated by BJH method using adsorption branches. [e]Prepared with 1 g of sodium polyacrylate. [f]Prepared with 2 g of the polymer. [g]Prepared with 3 g of the polymer.



3,4-Dihydrocoumarin — 3-(2-Hydroxyphenyl)propionic acid

Scheme 1. Hydrolysis reaction of 3,4-dihydrocoumarin.

The esterase was immobilized into silica microcapsules using silica precipitants such as NH_4Cl, $(NH_4)_2SO_4$, NH_4HCO_3 and $KHCO_3$.[9] A W/O (water/oil) emulsion, where the water phase was a sodium silicate solution with the esterase and the oil phase was n-hexane solution with Tween 85, was added to silica precipitant solutions to form W/O/W emulsion. Silica particles formed in the resulting solutions encapsulated the esterase directly (see supporting information). The profiles of microcapsules thus prepared are summarized in Table 1. While silica nanoparticles smaller than 100 nm may possibly be toxic to living organisms,[16,17] the particle sizes of all microcapsules were more than 100 nm as shown in Figure S4. It is thought that these silica microcapsules are ascertained to be safe materials. The hydrolysis reactions of 3,4-dihydrocoumarin by the esterase were estimated by monitoring the absorption intensities of the hydrolyzed product, 3-(2-hydroxyphenyl)propionic acid, at the wavelength of 270 nm (Scheme 1).[15] The time variations of the absorption of reaction solutions with various silica microcapsules are illustrated in Figure 1. When 0.3 mg of the original esterase (non-immobilized) was used, the absorption at wavelength of 270 nm was approximately stable at around intensity 1.5 (not shown in Figure 1). This meant that the hydrolysis reaction was immediately completed before the spectrophotometric measurement was started (within 1 min).

For balancing the amount of the esterase (its maximum content was up to 20 wt% in microcapsules as listed in Table 1), 1.5 mg of silica microcapsules immobilizing the esterase (**Cap1**–**Cap4**) were employed for the hydrolysis reaction. As shown in Figure 1, when a vacant microcapsule without the esterase (**Cap0**) was used (1.5

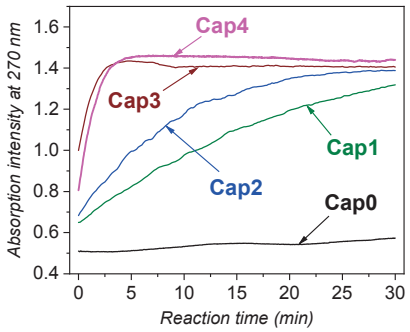


Figure 1. Time variation of the absorption intensity at wavelength of 270 nm in the hydrolysis reaction of 3,4-dihydrocoumarin using silica microcapsules immobilizing the esterase.

mg), the increase of the absorption intensity at 270 nm was slight even after 30 min. The vacant silica microcapsule was nearly inert for this reaction. On the other hand, the intensity elevations of the absorption were clearly observed when the microcapsules encapsulating the esterase were used (**Cap1**–**Cap4**). Thus, the hydrolysis reaction was catalyzed by the esterase included in the microcapsules. The intensity elevations at the early stage accurately displayed the enzymatic activities of these microcapsules. Rapid elevations of the intensity were found in the reactions using the microcapsules prepared with NH_4HCO_3 (**Cap3**) and $KHCO_3$ (**Cap4**). These reactions nearly completed within 5 min, because no further increases of the absorption intensity were observed. It appears that **Cap4** with larger pore volume and peak mesopore diameter than **Cap3** was superior to **Cap3**. On the other hand, the reactions using microcapsules prepared with NH_4Cl (**Cap1**) and $(NH_4)_2SO_4$ (**Cap2**) were slower. Since **Cap1** with the highest content of the esterase (18.7 wt%) showed the lowest reaction rate, the enzymatic activities of these microcapsules had no relation to the contents of the esterase. The difference of the activity between **Cap2** and **Cap3** that had the same pore volume is caused from the different distribution of mesopores shown in Figure S2. Higher volume of the mesopores of **Cap3** around 4 nm sufficiently larger than 3,4-dihydrocoumarin resulted in faster reaction rate. It is thought that the reaction rates were significantly influenced by the pore structures of silica microcapsules, because more porous **Cap4** had the highest activity. On the other hand, the esterase encapsulated into microcapsule was comparatively thermally stable, because the enzymatic activity was nearly unchanged when **Cap3** treated at 50 °C was used. However, the treatment at 80 °C moderately reduced the activity as indicated in Figure S5.

We reported before that the addition of suitable water-soluble polymers into a sodium silicate solution of W/O/W emulsion produces silica microcapsules bearing macropores.[10] Thus, this technique was applied to the immobilization of the esterase. In this study, sodium polyacrylate (Mw: ~2100) was used because of its high solubility to water. $KHCO_3$ was employed as precipitant in this preparation. From 1 to 3 g of the polymer was added to the solution of 29.8 g of sodium silicate, 4 g of the esterase and 13 mL of distilled water (**Cap5**–**Cap7**). As listed in Table 1, both the volumes and peak diameters of mesopore of these microcapsules (**Cap5**–**Cap7**) were lower than those of **Cap4**. Our previous paper[10] reported that mesopores of the microcapsules were expanded to macropores by the addition of water-soluble polymers, resulting in the decrease of their mesoporosity. In SEM images of Figure 2A, no pores observable by common SEM analysis were found in the

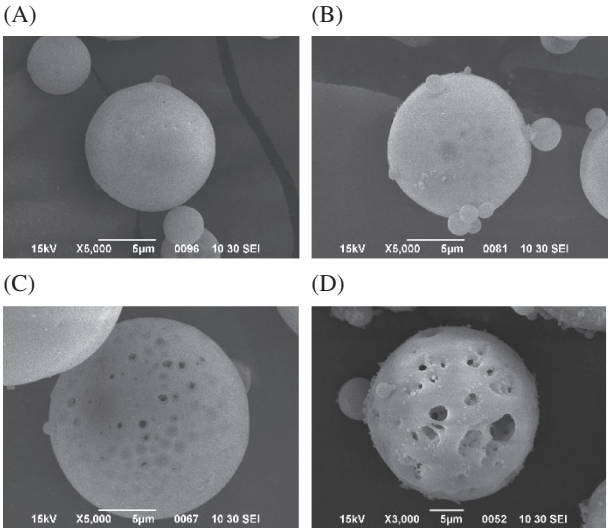


Figure 2. Scanning microscope images of silica microcapsules. (A) **Cap4**, (B) **Cap5**, (C) **Cap6** and (D) **Cap**7.

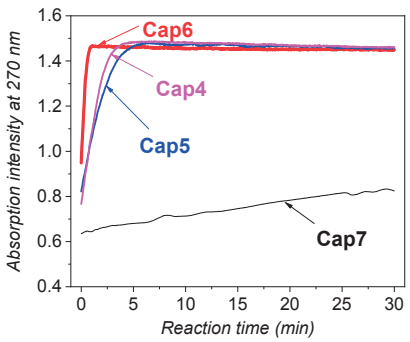


Figure 3. Time variation of the absorption intensity at wavelength of 270 nm in the hydrolysis reaction of 3,4-dihydrocoumarin using silica microcapsules encapsulating the esterase prepared with sodium polyacrylate.

microcapsules prepared without the polymer (**Cap4**). In the microcapsule **Cap5** prepared with 1 g of the polymer, cavities inside the microcapsule were observed as darkish spots (Figure 2B). On the other hand, in the microcapsules obtained using 2 g of the polymer (**Cap6**), macropores in the range of 100 to 500 nm were clearly observed (Figure 2C). Many cavities as darkish spots were also found. In the microcapsules obtained with 3 g of the polymer (**Cap7**), macropores larger than 1 μm were clearly observed (Figure 2D). Thus, the addition of the polymer fabricated macropores in microcapsules.

Figure 3 illustrates the reaction rate of the hydrolysis reaction of 3,4-dihydrocoumarin catalyzed by these silica microcapsules immobilizing the esterase. Although the microcapsule prepared with 1 g of the polymer (**Cap5**) showed a comparable reaction rate to **Cap4** that was obtained without the polymer, the macroporous microcapsule prepared with 2 g of the polymer (**Cap6**) displayed the highest reaction rate. The intensity elevation of the absorption at the wavelength of 270 nm was very rapid and the reaction was completed within 2 min. It is thought that the macropores in the microcapsule **Cap6** substantially facilitated the access of the reactant to the esterase, achieving this fast reaction. On the other hand, when the microcapsule obtained with 3 g of the polymer (**Cap7**) was used, the reaction was slow and unfinished even after 30 min.

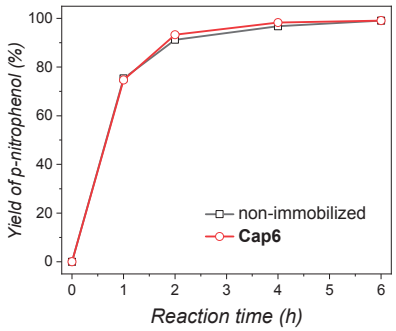


Figure 4. Time variations of the yield of *p*-nitrophenol from *p*-nitrophenyl acetate catalyzed by the non-immobilized esterase and the silica microcapsule immobilizing the esterase **Cap6** (per 5 mg of the esterase).

The silica microcapsule immobilizing the esterase bearing macropores from 100 to 500 nm (**Cap6**) had an excellent activity for the hydrolysis reaction.

As listed in Table 1, the content of the esterase in **Cap7** was estimated to be about 2.5 wt%, while all other microcapsules included more than 10 wt% of the esterase by TG-DTA analysis. The low activity of **Cap7** toward the hydrolysis of 3,4-dihydrocoumarin resulted from poor content of the esterase. It is likely that the macropore larger than 1 μm in **Cap7** is too large to encapsulate the esterase, and a great portion of the esterase was eliminated during the preparation process. On the other hand, since the size of macropores in **Cap6** was appropriate, this silica microcapsule contained sufficient amount of the esterase to show the highest performance in the hydrolysis reaction. The molecular size of the esterase (molecular mass: 60 kDa)[15] is likely to be comparable to that of bovine serum albumin (molecular mass: 66.4 kDa; molecular dimension $5.0 \times 7.0 \times 7.0\,nm^3$),[13] which is greatly smaller than the macropore of **Cap6**. The recycling of **Cap6** was examined as illustrated in Figure S6. Although the enzymatic activity gradually decreased with recycling, the hydrolysis reaction was completed after about 10 min even in the tenth reaction (ninth recycling). Despite **Cap6** having enormously larger macropores than the esterase, the leaching of esterase by recycling scarcely occurred and it is recyclable for the reaction.

Although the reaction rate of the hydrolysis of 3,4-dihydrocoumarin by the macroporous silica microcapsule **Cap6** was definitely quick, the rate by non-immobilized esterase is much faster because this enzyme is specific to the reaction. Then, we attempted another reaction, the hydrolysis of *p*-nitrophenyl acetate, which is well examined for the evaluation of esterase enzymes.[18] The reaction rates of this hydrolysis were compared between the immobilized and non-immobilized esterase. 5 mg of the non-immobilized esterase and 30 mg of the silica microcapsule encapsulating the esterase **Cap6** were used for balancing the esterase contents between them. In this case, the reaction was monitored by the intensity of the absorption of *p*-nitrophenol as hydrolyzed product at the wavelength of 400 nm. As illustrated in Figure 4, the time variation of the yield of *p*-nitrophenol in the reaction with **Cap6** was closely overlapped with that of the non-immobilized esterase. The esterase immobilized in the macroporous silica microcapsule had nearly the same activity as the non-immobilized esterase for the hydrolysis reaction of *p*-nitrophenyl acetate, because the large macropore of the silica microcapsule **Cap6** scarcely reduced the reaction rate by the esterase. In this reaction, it is thought that the reaction was controlled not by the access of the reactant to the esterase (diffusion-controlled reaction) but by the reactivity of the esterase (reaction-controlled reaction).

In conclusion, an esterase obtained from *Acinetobacter calcoaceticus* F46 was successfully immobilized into silica microcapsules. The esterase encapsulated in the silica microcapsules had hydrolysis activity toward esters. The reaction rate of the hydrolysis of 3,4-dihydrocoumarin by the esterase was mainly influenced by the pore properties of the silica microcapsules. A silica microcapsule that had macropores up to 500 nm showed the fastest reaction rate. The wide macropore facilitating the access of the reactant to the esterase promoted the reaction. This macroporous silica microcapsule nearly had an enzymatic activity equivalent to the non-immobilized esterase in the hydrolysis reaction of *p*-nitrophenyl acetate. The preparation technique of macroporous silica microcapsules will contribute to the advancement of the technology of enzyme immobilization.

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Supporting Information

Experimental procedures, nitrogen sorption isotherms, the BJH mesopore distribution, SEM images, and particle size distribution of silica microcapsules. This material is available on https://doi.org/10.1246/bcsj.20200101.

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