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Subtilisin-Catalyzed Transesterifications in the Presence of Iron Oxide Nanoparticles in Organic Solvent: Dramatic Catalytic Improvement†

Jong Kyung Bang, Sang Oh Jung, Yunwoong Kim, and Mahn-Joo Kim*

Department of Chemistry, Pohang University of Science and Technology, Pohang 790-784, Korea
**E-mail mjkim@postech.ac.kr*
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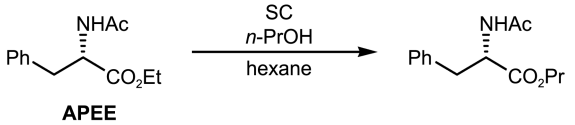
Enzymatic transformations in organic media provide a useful methodology for the synthesis of a wide range of enantiomeric compounds.[1] In spite of remarkable advances in this field, the enzymes still suffer from a limitation that they display significantly reduced activities in organic media, which are often several orders of magnitude lower than aqueous counterparts. In the past decades, intensive studies have been done to develop methods for enhancing enzymatic activity in organic media. A useful approach is the lyophilization of enzyme in the presence of excipients such as lyoprotectants, polymers, and salts.[2] For example, subtilisin lyophilized in the presence of KCl salts (98%, W/W) displayed three orders of magnitude higher activity than its salt-free counterpart in organic solvent.[3] The salt–activated subtilisin as the catalyst for synthetic applications, however, has some disadvantages that a relatively large volume of enzyme–salt mixture should be used and its recovery for reuse is troublesome. We herein wish to report a practical approach using iron oxide nanoparticles (IONs) as the additive for high activity and easy recovery of subtilisin.

Subtilisin Carlsberg (SC, *Bacillus licheniformis*) displays (*S*)–enantioselectivity toward simple secondary alcohols,[4] which is complementary to the (*R*)-enantioselectivity of lipase. Recently, our group and others demonstrated that SC has great potential as the catalyst for the dynamic kinetic resolution (DKR) of secondary alcohols[5a,b] and primary amines.[5c] DKR is a powerful tool for the transformations of racemates to single enantiomers because it can provide high yields and excellent enantiopurities, both approaching 100%. For its wider applications in DKR, SC should be readily activated and recovered. During our testing of IONs as a magnetically recoverable supporter for SC,[6] we found that IONs activated SC dramatically when they were co-suspended with enzyme powder in organic solvent.

In typical procedures, native SC in powder form was prepared by lyophilizing a commercial SC (99%, W/W) in the presence of phosphate buffer (1%, W/W; pH 7.8). The native SC was mixed physically with IONs[7] (average size 12 nm) at several different weight ratios in a vial. The native and IONs–SC samples were then tested as the catalysts for the transesterification of *N*-acetyl-L-phenylalanine ethyl ester (APEE) in hexane (Scheme 1). The specific activity of the native SC, measured in the presence of 10 mM APEE at 25 °C, was 4.5 mU (U = mmoles of product per mg of enzyme per hour). Surprisingly, the IONs–SC preparations displayed three order of magnitude higher activities than the native SC (Figure 1). The specific activity of IONs–SC was *ca* 4 U at 20 : 1 weight ratio, increased gradually with increasing the relative weight of IONs, and reached 9.52 U at 99 : 1 weight ratio. Data from Table 1 indicate that the catalytic efficiency (V_{max}/K_m) of IONs–SC at 99 : 1 weight ratio is 2470-fold higher than that of its IONs–free counterpart and such a dramatic enhancement is largely a result of an increase in V_{max}.

To compare IONs with KCl salts in activating SC, we



Scheme 1. Subtilisin Carlsberg (SC)-catalyzed transesterification of *N*-acetyl-L-phenylalanine ethyl ester (APEE).

†This paper is dedicated to Professor Eun Lee on the occasion of his honourable retirement.

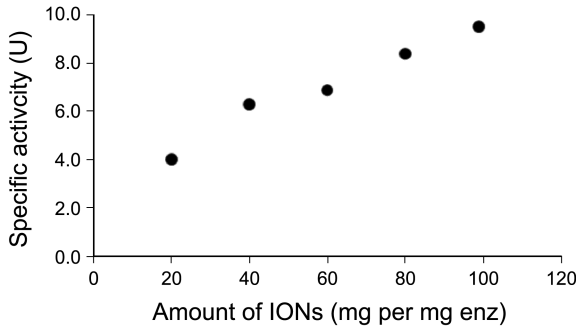


Figure 1. The specific activity of IONs-activated subtilisin Carlsberg (SC) against the amount of IONs added in the transesterifications of *N*-acetyl-L-phenylalanine ethyl ester (APEE).

Table 1. Kinetic Parameters of Subtilisin Carlsberg (SC).[a]

enzyme	V_{max} (U)[b]	K_m (mM)	V_{max}/K_m	activation factor
native	2.82×10^{-2}	25.3	1.11×10^{-3}	
IONs-SC (99 : 1, W/W)	13.2	4.92	2.74	2470

[a] The kinetic parameters were obtained from the Lineweaver-Burk plot. [b] U = mmoles of product per mg of protein per hour

prepared two enzyme samples containing 98%(W/W) IONs and 98%(W/W) KCl, respectively. The 98% IONs–SC sample was prepared by simply mixing a lyophilyzed SC sample (2 %, W/W; 1 % SC plus 1 % phosphate buffer) with IONs (98%, W/W). The 98% KCl–SC sample was prepared by lyophilizing SC (1%, W/W) in the presence of phosphate buffer (1%, W/W) and KCl salts (98%, W/W) according to the literature procedure.[3b] It was observed that the specific activities of 98% IONs–SC and 98% KCl–SC were 7.52 U and 7.32 U, respectively. This observation indicates that IONs are as good as KCl salts in activating SC. On the other hand, KCl salts have been known to be ineffective as the activators in case they are added to organic solvent containing enzyme powder.[3b] In contrast, the activation effect of IONs remained unaltered when they were added to enzyme powder in organic solvent.

We speculate that two factors would contribute to such a dramatic activation of SC in the presence of IONs. First, IONs have a large surface-to-volume ratio. SC molecules are expected to be physically adsorbed on IONs by mixing and then dispersed on the large surface of IONs by shaking during reaction, thus lowering mass transfer barrier. Second, IONs contain water or hydroxyl groups on their surface. The water content of IONs, determined by Karl–Fisher titration method in this work, was *ca* 3.5%(W/W). Water or hydroxyl groups on the surface of IONs might provide aqueous–like microenvironments for more efficient enzymatic catalysis.

The effect of water on the activity of SC was examined with the addition of water to the solution containing IONs-free SC. The activity of SC increased gradually with increasing the amount of added water (Figure 2, open circle). In contrast, the activity of SC in the presence of IONs increased more rapidly with increase of water content by the addition of IONs. Furthermore, its activity extrapolated at zero water content is 3.05 U, corresponding to about 670-fold activation. These results suggest that a major contributing factor to the activation of SC by IONs is not water in IONs but the dispersion of enzymes on the large surface of IONs. At present, no theoretical rationale is available for such a huge activation by IONs-induced dispersion. However, we could hypothesize that SC is poorly active in its aggregation state because the active sites of enzyme molecules are covered by neighboring enzyme molecules and therefore unavailable to substrates, but, as enzymes are deaggregated by their dispersion on the large surface of IONs, the active sites are opened and become readily available to substrates, thus resulting in a dramatic increase in activity.

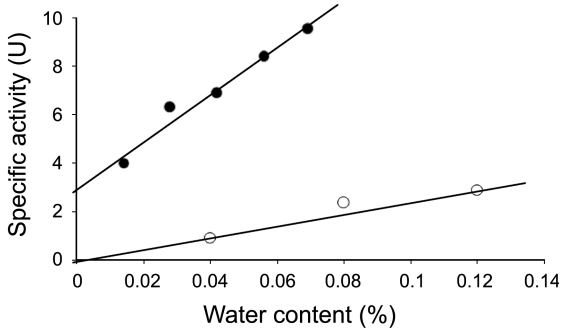


Figure 2. Plot of specific activities of IONs-SC (•) and IONs-free SC (○) against water content in solution. The water contents represent the amounts of water in 5 mL of solution. In case of IONs-SC, the amounts of water in solution were calculated using the water content of IONs.

In summary, we have demonstrated that IONs enhance dramatically the activity of SC. IONs are as effective as well–known KCl salts, but the former have a big advantage over the latter that it is readily recovered for reuse. The procedure for the preparation of IONs–SC is simple and requires no chemical modifications on both enzyme and IONs. Further studies on the applications of IONs for activating other proteases as well as the synthetic applications of IONs-SC composites are currently under way in our laboratory.

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7. IONs were prepared according to the literature method (Abu-Reziq, R.; Wang, D.; Post, M. L.; Alper, H. *Adv. Synth. Catal.* **2007**, *349*, 2145) and their sizes were determined by transmission electron microscopy (TEM) analysis. IONs were mixed with SC using a spatula in a vial.