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Enantioselective Ammonolysis of Phenylglycine Methyl Ester with Lipase–Pluronic Nanoconjugate in Tertiary Butanol

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Abstract Asymmetrical ammonolysis of (*R*)- and (*S*)-phenylglycine methyl ester was carried out by using a lipase (CALB)–polymer (Pluronic) nanoconjugate as the catalyst, displaying a 11-fold increased catalytic rate compared to the free CALB in tertiary butanol.

Keywords Enzymatic catalysis · Nanoparticles · Enzymes

1 Introduction

R-Phenylglycine and its derivatives are key intermediates in the synthesis of penicillin and cephalosporin antibiotic [1, 2], which are produced by using the classical resolution via diastereomeric salt crystallization and the enantioselective hydrolysis of corresponding hydantion [3]. The unsatisfactory selectivity and yield of above procedures result in the implementation of laborious separation and purification steps and hence the excess mass and energy consumption and waste generation. While the enzymatic ammonolysis is reported for the green synthesis of chiral synthons [4, 5], the enzymatic ammonolysis of racemic phenylglycine methyl esters for the production of *R*-phenylglycine amide is challenged by the extremely low apparent activity of enzyme in organic solvents, being one to several orders of magnitude lower than that in aqueous solution [6]. One major reason underpinned the low apparent enzymatic activity is the poor solubility of enzyme in the organic solvents, which leads to the aggregation of enzyme into a rigid form that not only hinders the conformational transition requested for the catalysis but also reduces the accessibility to its substrate. On the other hand, the ammonium salt as ammonia donor in the enzymatic ammonolysis reaction has very limited solubility in organic solvents, which further hinders the contact and subsequent uptake by the enzyme aggregates.

Nanostructured enzyme catalysts [7–10] such as enzyme nanoparticles [11, 12], enzyme nanogels [13–17], and flower-like enzyme-inorganic hybrid crystals [18–22] have shown novel ways to enhance enzymatic reactions in both aqueous and non-aqueous media. For example, by conjugation with an amphiphilic polymer such as Pluronic, the solubilization of enzyme catalysts in organic solvents is realized. This facilitates the access of the substrate to the active site of enzyme and elevates the apparent enzymatic activity by several hundred folds compared to its native counterpart [23]. This thus encourages us to explore the potential of lipase–Pluronic conjugate for the enantioselective ammonolysis of phenylglycine methyl ester in organic media (Scheme 1).

2 Experimental

The *Candida antarctica* lipase B (CALB)–Pluronic conjugate was synthesized according to Ref. [23]. The hydroxyl group of Pluronic F-127 was first oxidized to aldehyde group by using Dess–Martin periodinane as the oxidizing reagent. The obtained aldehyde-functionalized

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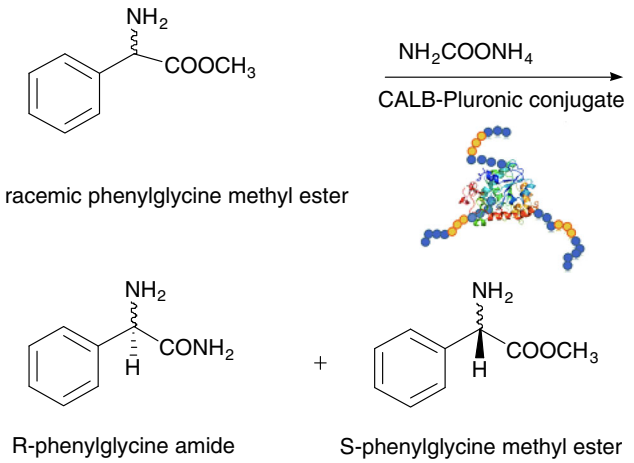

Scheme 1 Ammonolysis of phenylglycine methyl ester in organic media catalyzed by lipase–Pluronic nanoconjugate

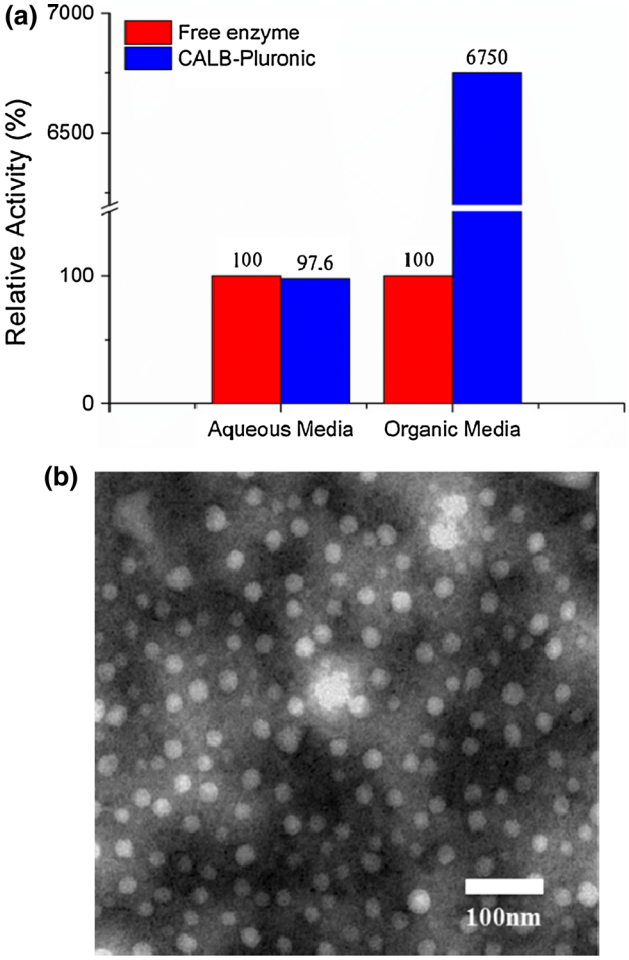

Fig. 1 **a** The activities of CALB–Pluronic conjugate compared to native lipase in aqueous and organic media; **b** TEM image of the CALB–Pluronic conjugate

Pluronic was dissolved in phosphate buffer (10 mM, pH 7.0) and mixed with aqueous solution of CALB (5–10 mg/mL) (the molar ratio of the aldehyde group to the amine group of protein is 1.1:1). After 2 h reaction, $NaCNBH_3$ was added to the mixture to reduce the Schiff base, followed by dialysis in phosphate buffer solution (10 mM, pH 7.0) to remove unreacted reagents. Finally, the powder of CALB–Pluronic conjugate was obtained by lyophilization. For transmission electron microscopy (TEM) measurements, the dry powder of the lipase–Pluronic conjugate obtained by lyophilization was dissolved in toluene (10 μg/mL of protein concentration). Ten microlitre of this solution was placed on the carbon-coated grid. After toluene evaporated, ten μL of sodium phosphotungstate aqueous solution (1 %, pH 7.0) was applied to stain the sample. The excess of the liquid was removed after 2 min and the sample was dried for 24 h at room temperature before making TEM measurements.

3 Results and Discussion

The relative hydrolytic activity of the CALB–Pluronic conjugate determined with 4-nitrophenyl butyrate (*p*-NPB) as the substrate was ~97 % with reference to free CALB. In toluene, using hexanoic acid and *n*-butyl alcohol as the substrates, the apparent esterification activity of the conjugate in toluene was increased by ~67-fold in comparison to free CALB (Fig. 1a) at the same protein amount. TEM image of the CALB–Pluronic conjugate showed that it is presented in the form of nanospheres with diameters from 10–50 nm (Fig. 1b). It is likely that the hydrophobic PPO block of Pluronic extended into the organic phase, and meanwhile the hydrophilic PEO blocks stayed around the conjugated protein, which resulted in the formation of the self-assembled conjugates with sizes from 10 to 50 nm.

The catalytic performance of the CALB–Pluronic nanoconjugate in the ammonolysis of phenylglycine methyl ester was investigated in comparison with free CALB. In a typical experiment, 66 mg of the racemic phenylglycine methyl ester and 125 mg of ammonium carbamate as the ammonia donor [2, 24] were added in 2 mL of tertiary butanol (TBA). Then, 20 mg of the CALB–Pluronic nanoconjugate (or free CALB containing the equivalent amount of protein) was added to the mixture followed by gentle shanking at 150 rpm, 30 °C. At intervals, samples were taken for HPLC analysis to determine the conversion of the substrate and the enantiomer excess (ee) value for the enzymatic reaction.

As shown in Fig. 2, at the same protein amount, the reaction catalyzed by CALB–Pluronic nanoconjugate at 30 °C reached a conversion of ~60 % in 30 h while that catalyzed by free CALB below 20 %. Calculated from the

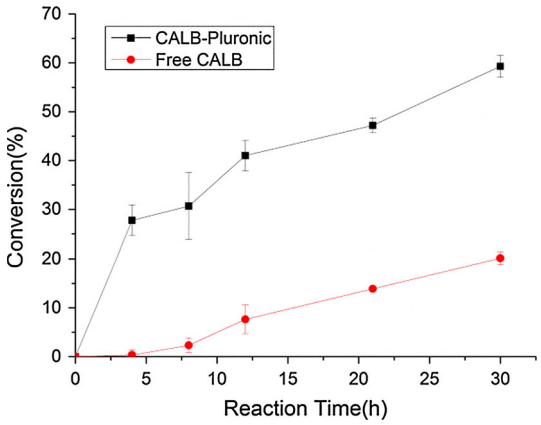

Fig. 2 Conversions of phenylglycine methyl ester in the ammonolysis reaction in TBA catalyzed by CALB–Pluronic conjugate and free CALB

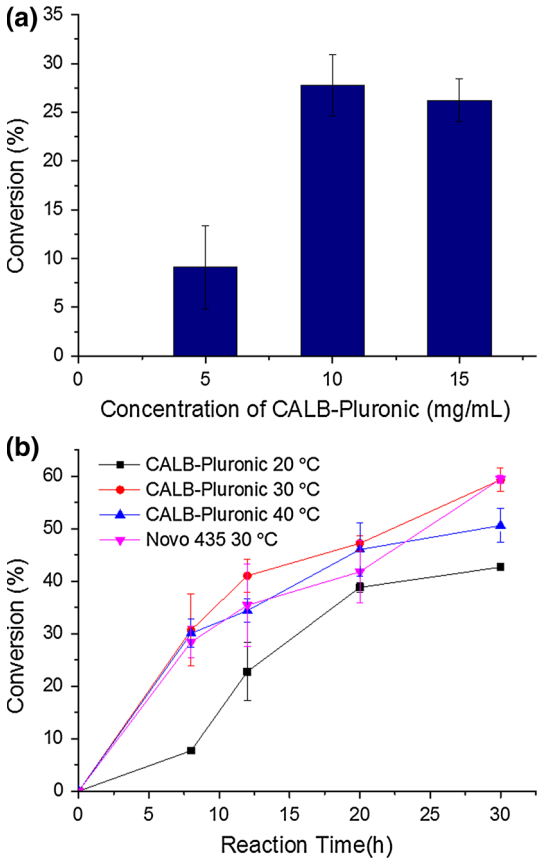

Fig. 3 **a** Conversions of the substrate catalyzed by the conjugate with different concentrations, 4 h, 30 °C, in TBA. **b** Conversions of the substrate catalyzed by the conjugate at different temperatures

initial reaction rate, the apparent activity of the conjugate was about 11-fold higher than that of the free CALB in this specific reaction.

Table 1 Comparison of the conversion and selectivity of CALB–Pluronic conjugate with Novo 435 in different solvents

Solvent	Novo 435[a]	CALB–Pluronic[a]
MTBE		
Conversion (%)	53.7	53.9
ee (%)	54.9	50.7
TBA		
Conversion (%)	41.8	62.9
ee (%)	42.9	64.8

[a] *The reaction condition* 20 mg Novo 435 (or CALB–Pluronic containing same amount of protein), 2 mL MTBE (or TBA), 66 mg racemic phenylglycine methyl ester, 125 mg ammonium carbamate, 30 °C, 200 rpm

To optimize the reaction catalyzed by CALB–Pluronic nanoconjugate, the effects of the reaction parameters such as the conjugate loading and temperature on the conversion were examined.

As shown in Fig. 3a, the increase in the conjugate concentration from 5 to 10 mg/mL elevated the conversion from ~10 to 28 % in 4 h. Further increase in the concentration contributed less to the conversion. Furthermore, as shown in Fig. 3b, at which the conjugate concentration was 10 mg/mL, the elevation of reaction temperature from 20 to 30 °C accelerated the reaction significantly while further elevation to 40 °C contributed to a marginal increase in the reaction rate. With the above mentioned efforts, the optimal temperature and conjugate concentration was determined as 30 °C and 10 mg/mL, respectively. Under this condition, the final conversion of the substrate reached ~60 % in 30 h. For the purpose of comparison, the above reaction was also conducted using a commercial immobilized CALB (Novo 435), which has excellent activity and stability in organic media [25–27]. As shown in Fig. 3b, the CALB–Pluronic nanoconjugate showed similar catalytic activity as Novo 435.

The enantio-selectivity of CALB–Pluronic nanoconjugate in the ammonolysis of racemic phenylglycine methyl ester in organic solvents was examined in comparison with Novo 435 as well (Table 1). In TBA, the ee value of the reaction catalyzed by the conjugate reached 64.8 % at a final conversion of 62.9 %, which is higher than that with Novo 435 (42.9 %). In principle, the theoretically highest conversion of the substrate in an enatio-ammonolysis of racemic substrate is 50 % at a 100 % selectivity. The conversions above 50 % (Table 1) observed in both Novo 435 and CALB–Pluronic conjugate, indicated the non-perfect selectivity of the conjugate and Novo 435, which led to the ammonolysis of *S*-type substrate and thus gave conversions over 50 %. In addition to TBA, methyl tertiary butyl ether (MTBE) was also examined as the solvent for the lipase-catalyzed ammonolysis reaction. In MTBE,

CALB–Pluronic nanoconjugate performed similarly as Novo 435 in terms of both conversion and selectivity (Table 1).

4 Conclusion

In summary, we prepared a CALB–Pluronic nanoconjugate for the ammonolysis of phenylglycine methyl ester in organic media. For this reaction, the CALB–Pluronic conjugate displayed a 11-fold higher apparent activity compared to the free CALB in TBA. At an optimized condition, the conversion and ee value of the reaction catalyzed by the conjugate reached 62.9 and 64.8 %, respectively. The high activity of CALB–Pluronic conjugate demonstrated by this study indicates its potential applications in the lipase-catalyzed ammonlysis reactions for the production of chiral amides which are important intermediates for fine chemical and pharmaceutical industry.

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