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The lipase-catalyzed asymmetric C–C Michael addition

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ABSTRACT

The example of enzyme-catalyzed asymmetric C–C Michael addition was observed using Lipozyme TLIM (immobilized lipase from *Thermomyces lanuginosus*) in organic medium in the presence of water. This biocatalysis is applicable to the Michael additions of a wide range of 1,3-dicarbonyl compounds and cyclohexanone to aromatic and heteroaromatic nitroolefins and cyclohexenone. The enantioselectivities up to 83% ee and yields up to 90% were achieved. The enzyme can be reused for three cycles.

1. Introduction

C–C bond forming reactions are one of the mainstays of organic chemistry. In this field the Michael reaction has numerous applications in synthetic chemistry [1–5]. The Michael reactions were classically catalyzed by bases or suitable combinations of amines and carboxylic or Lewis acids under homogeneous conditions. The employment of these bases in the reactions encounters environmental problems [6]. Therefore, there has been increasing attention on the design and use of environmentally compatible catalysts [6].

On the other hand, biocatalysis has received great attention as an efficient and green tool for the synthesis of pharmaceutical, industrial and agricultural chemicals and intermediates [7–10]. Some elegant works on enzyme-catalyzed Michael addition have been reported, for example lipase catalyzed formation of C–C [11–14], C–N [15–27], C–O [15], C–S [15,26,27] and D-aminoacylase catalyzed formation of C–C bond via Michael addition [14,28]. However, to the best of our knowledge, enzyme-catalyzed asymmetric C–C Michael addition has never been reported. Although some efforts have been made by other groups [12–14,29], no enantioselectivity has been observed in this biotransformation. Herein, we wish to report the first discovery that Lipozyme TLIM (immobilized lipase from *Thermomyces lanuginosus*) can catalyze asymmetric C–C Michael addition in organic medium in the presence of water, and the enantioselectivities up to 83% ee and yields up to 90% were achieved. The effects of organic solvent, temperature and water content on the enzymatic Michael addition were evaluated in detail. The catalytic specificity of immobilized lipase was demonstrated by the control experiments. This asymmetric Michael addition activity of Lipozyme TLIM provided a novel case of unnatural activities of existing enzymes.

2. Experimental

2.1. Materials

Lipozyme TLIM (immobilized lipase from *Thermomyces lanuginosus*, EC 3.1.1.3, 0.25 U/mg) was purchased from Novozymes Biotechnology Co., Ltd. (Tianjin, PR China). Lipase from porcine pancreas (≥3000 U/mg enzyme activity pH 7.7, 37 °C) was purchased from Shanghai Lanji Technology Development Co., Ltd. (Shanghai, PR China). Lipase from *Candida cylindracea* (4.28 U/mg), lipase from porcine pancreas type II (100–400 U/mg protein, one unit will hydrolyze 1.0 microequivalent of fatty acid from triacetin in 1 h at pH 7.4 at 37 °C) and Amano lipase M from *Mucor javanicus* (≥10,000 U/g enzyme activity, pH 7.0, 40 °C) were purchased from Sigma-Aldrich Trading Co., Ltd. (Shanghai, PR China). Lipase AYS "Amano" *Candida rugosa* (≥30,000 U/g enzyme activity, one unit is the amount of enzyme that releases 1 μmol of fatty acid per 1 min at pH 7.0), Lipase PS "Amano" SD from *Burkholderia cepacia* (≥23,000 U/g enzyme activity, pH 7.0–8.0) and Lipase AK "Amano", from *Pseudomonas fluorescens* (≥20,000 U/g enzyme activity, pH 8.0, 60 °C) were a gift from Amano Enzyme Inc. (Shanghai, PR China). Unless otherwise noted, all reagents were obtained from commercial suppliers and were used without further purification.

2.2. Analytical methods

All reactions were monitored by thin-layer chromatography (TLC) with Haiyang GF254 silica gel plates. Flash column

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chromatography was carried out using 100–200 mesh silica gel at increased pressure. The ^{1}H NMR spectra were recorded with TMS as internal standard on a Bruker AMX-300MHz spectrometer. Chemical shifts were expressed in ppm and coupling constants (*J*) in Hz. HPLC was carried out using Chiralcel AD-H, OD-H, AS-H columns. All the known products were characterized by comparing the ^{1}H NMR with those reported in the literature.

2.3. Typical procedure for the enzyme catalyzed Michael addition (synthesis of product **a**)

A mixture of β-nitrostyrene (3.0 mmol), ethyl acetoacetate (1.0 mmol), Lipozyme TLIM (200 mg) in DMSO (5 ml) and deionized water (0.5 ml) was stirred for 75 h at 35°C. Enzyme was filtered off to stop the reaction. CH_2Cl_2 was used to wash the filter paper to assure that products obtained were all dissolved in the filtrate. Then 30 ml of water was added to the filtrate, and the filtrate was extracted with CH_2Cl_2. The organic phase was dried over anhydrous Na_2SO_4, and the solvents were removed under reduced pressure. The mixture was purified by flash chromatography using EtOAc/petroleum ether (1:6 v/v) as eluent to afford the product **a** as a white solid, 220 mg (79%, 65:35 dr, 16%/>99% ee).

2.4. The procedure for recycling and reusing of Lipozyme TLIM

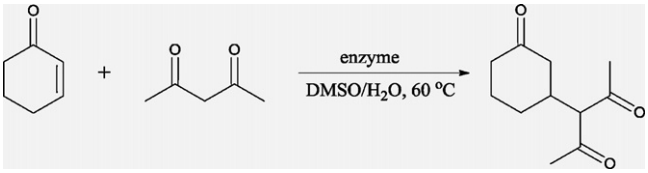
A mixture of Lipozyme TLIM (200 mg), DMSO (5 ml), thienyl nitroolefin (3.0 mmol), acetylacetone (1.0 mmol) and deionized H_2O (0.5 ml) was stirred at 35°C. The same procedure as described above was applied to obtain the product. The enzyme was recovered by filtrating from the reaction system, washed twice with ethanol and three times with acetone, and dried in the air at r.t. The recovered enzyme was then used in the subsequent Michael reaction without adding any new enzyme for the same stoichiometry of substrates as that in the first cycle.

3. Results and discussion

Based on the concept that in organic solvents, enzymes acquire remarkable properties such as enhanced stability, altered substrate and enantiomeric specificities, molecular memory, and the ability to catalyze unusual reactions which are impossible in aqueous media [30], we committed ourselves to screening of new activities of existing enzymes in organic solvents. In our initial study, a preliminary enzyme screen was performed (Table 1) to find out the efficient catalyst for C–C Michael addition. The reaction between cyclohexenone and acetylacetone was used as a model reaction, and eight lipases were examined. As shown in Table 1, the best yield of 78% was achieved using lipase from porcine pancreas, but it gave inferior enantioselectivity with 4% ee (Table 1, entry 1). The good yield of 75% with the best ee of 17% was obtained using Lipozyme TLIM (Table 1, entry 2). In addition, the other tested lipases also showed different degrees of catalytic activity, however, no better enantioselectivity was obtained (Table 1, entries 3–8). Therefore, we decided to investigate Lipozyme TLIM in Michael addition with respect to the synthetic potential and stereochemistry of the products.

Having established the optimal catalyst, the solvent screen was performed (Table 2) to find out the optimal solvent for this biotransformation. The reaction in THF gave the best yield of 74%, while the yields of 69% and 68% were obtained respectively in DMSO and DMF (Table 2, entries 1–3). The other tested solvents gave lower yields (Table 2, entries 4–10). No clear correlation between solvent polarity and the enzyme activity was observed. We also investigated the enantioselectivity of this enzymatic reaction in different solvents, and the best ee value of 17% was obtained from DMSO. Interestingly, we found that enzyme enantioselectivity in organic media

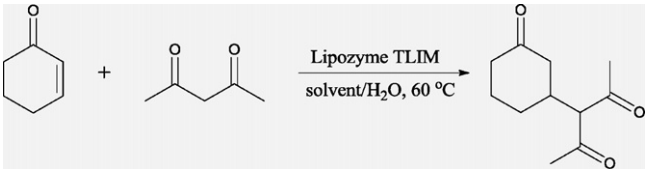
Table 1
Enzyme screening for Michael addition of cyclohexenone and acetylacetone[a].



Entry	Enzyme	Time (h)	Yield (%)[b]	ee (%)[c]
1	Lipase from porcine pancreas	75	78	4 (S)
2	Lipozyme TLIM	75	75	17 (S)
3	Lipase from *Candida cylindracea*	75	63	11 (S)
4	Lipase from porcine pancreas type II	75	50	5 (S)
5	Lipase AYS "Amano" *Candida rugosa*	90	22	5 (S)
6	Lipase PS "Amano" SD from *Burkholderia cepacia*	90	13	7 (S)
7	Amano lipase AK, from *Pseudomonas fluorescens*	90	35	2 (S)
8	Amano lipase M, from *Mucor javanicus*	90	34	0

[a] The reaction was conducted using acetylacetone (100 mg, 1.0 mmol), enzyme (400 mg) cyclohexenone (194 mg, 2.0 mmol), deionized water (1 ml) and DMSO (5 ml) at 60 °C.
[b] Yield of the isolated product after chromatography on silica gel.
[c] Enantiomeric excess and absolute configuration were determined by HPLC analysis using a chiral column [42].

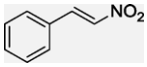
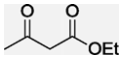
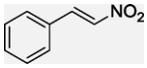
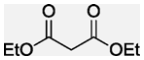
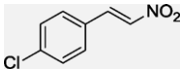
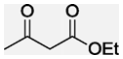
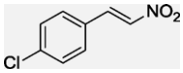
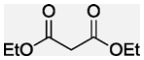
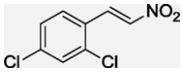
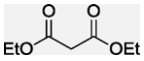
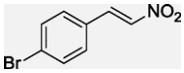
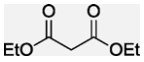
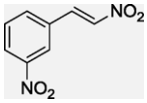
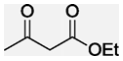
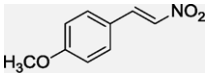
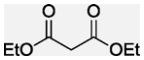
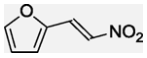
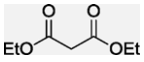
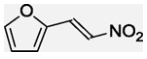
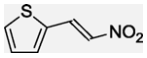
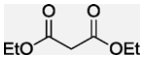
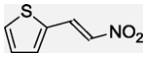
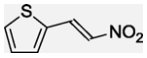
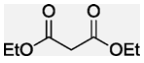
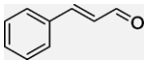
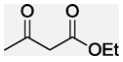
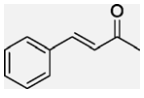
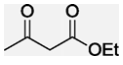
Table 2
Michael reaction of cyclohexenone and acetylacetone catalyzed by Lipozyme TLIM in different solvents[a].



Entry	Solvent	Time (h)	Yield (%)[b]	ee (%)[c]
1	THF	48	74	5 (*R*)
2	DMSO	48	69	17 (*S*)
3	DMF	48	68	5 (*S*)
4	EtOH	48	56	7 (*R*)
5	H_2O	48	55	2 (*S*)
6	1,4-Dioxane	48	45	8 (*R*)
7	n-Hexane	48	38	7 (*S*)
8	Toluene	48	32	4 (*R*)
9	Ethylether[e]	48	20	2 (*R*)
10	Acetonitrile	48	19	11 (*S*)
11	DMSO (no enzyme)	75	n.d.[d]	
12	DMSO (Lipozyme TLIM inhibited with PMSF[f])	84	n.d.[d]	

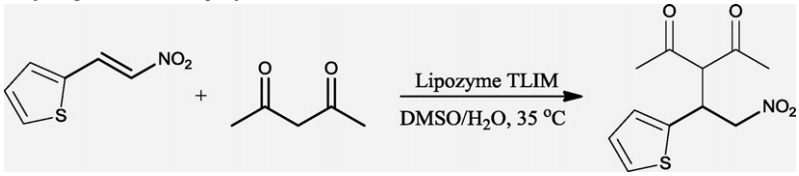
[a] All reactions were carried out using acetylacetone (100 mg, 1.0 mmol), Lipozyme TLIM (400 mg) cyclohexenone (194 mg, 2.0 mmol), deionized water (1 ml) and organic solvent (5 ml) at 60 °C.
[b] Yield of the isolated product after chromatography on silica gel.
[c] Enantiomeric excess and absolute configuration were determined by HPLC analysis using a chiral column [42].
[d] n.d.: Not detected.
[e] Under reflux.
[f] Pre-treated with PMSF (phenylmethanesulfonyl fluoride) at 25 °C for 24 h.

Table 3
Investigation of substrate scope in Lipozyme TLIM catalyzed Michael addition[a].

Entry	Acceptor	Donor	Product	Time (h)	Yield (%)[b]	dr[c]	ee (%)[d] (config.)[e]
1			a	75	79	65:35	16/>99[f]
2			b	72	53		9 (*S*)
3			c	70	90	60:40	n.d.[g]
4			d	72	30		8 (*R*)
5			e	72	75		52 (*R*)
6			f	72	45		7 (*S*)
7			g	72	65	60:40	n.d.[g]
8			h	72	50		8 (*S*)
9			i	72	56		3 (*R*)
10			j	110	60	84:16	7/25[f]
11			k	72	68		43[f]
12			l	125	85		83[f]
13			m	168	73	98:2	23/>99[f]
14			n	96	75		30 (*S*)
15			o	72	32		n.d.[g]
16			p	120	n.d.[h,i]		
17			q	240	n.d.[h]		
18			r	240	trace		
19			s	264	n.d.[h]		

[a] The reaction was conducted using the donor (1.0 mmol), the acceptor (3.0 mmol), Lipozyme TLIM (200 mg), H_2O (0.5 ml) and DMSO (5 ml) at 35 °C.
[b] Yield of the isolated product after chromatography on silica gel.
[c] Dr was determined by ^1H NMR and HPLC analysis using a chiral column.
[d] Enantiomeric excess was determined by HPLC analysis using a chiral column.
[e] Absolute configuration was determined by comparing the retention time of HPLC of adduct with that of literature data [43–49].
[f] Absolute configuration was not determined.
[g] n.d. = Not determined.
[h] n.d. = Not detected.
[i] No Michael product was detected; only a Knoevenagel product was obtained.

Table 4
Recycling and reuse of Lipozyme TLIM[a].



Cycle	Time (h)	Yield (%)[b]	ee (%)[c]
1	120	85	83
2	120	70	62
3	120	52	5
4	120	18	0

[a] Reaction conditions: Acetylacetone (1.0 mmol), thienyl nitroolefin (3.0 mmol), DMSO (5 ml), deionized H_2O (0.5 ml), Lipozyme TLIM (200 mg for the first cycle) at 35 °C.
[b] Yield of the isolated product after chromatography on silica gel.
[c] ee was determined by HPLC analysis using a chiral column.

depends on the solvent, and even reversal of enzyme enantioselectivity upon a change in the solvent was observed (Table 2). There have been numerous reports regarding this phenomenon [31–41]; however, in the most of them, no mechanistic explanation of the observed behavior was offered. This observation may be attributed to specific interactions between the solvent and the lipase. Based on the results of solvent screen, DMSO was chosen as the optimum solvent for the enzymatic Michael reaction. In addition, no product was detected in the blank experiment (Table 2, entry 11). Besides, it was experimentally verified that the inhibition of Lipozyme TLIM caused a complete disruption of the catalytic activity of the enzyme (Table 2, entry 12). The control experiments clearly indicated that Lipozyme TLIM had a specific catalytic effect on Michael addition.

To further optimize experimental conditions, we examined the effects of water content, temperature, molar ratio of substrates and enzyme loading on the yield of Michael reaction. As a consequence, 0.1 water content (water/solvent, v/v), 35 °C, 3:1 (acceptor/donor) and enzyme concentration of 36 mg/ml were chosen as the optimal conditions.

To test the substrate generality of Lipozyme TLIM catalyzed Michael addition, different aromatic and heteroaromatic nitroalkenes, cyclohexenone, as well as other α,β-unsaturated aldehydes and ketones were used as the acceptors to react with 1,3-dicarbonyl compounds and cyclohexanone under the optimized conditions. The results were summarized in Table 3. It can be seen that a wide range of substrates could participate in the reaction. Both electron-donating and electron-withdrawing functionalities were compatible (Table 3, entries 3–8). The best yield of 90% was obtained for the reaction of 4-chloro-β-nitrostyrene and ethyl acetoacetate (Table 3, entry 3). It was observed that ethyl acetoacetate was more reactive than diethyl malonate as the Michael donor in the enzymatic reaction, probably due to the easy formation of enolate. In addition, furyl nitroalkene reacted with diethyl malonate as well as cyclohexanone to give the corresponding products in acceptable yields (Table 3, entries 9 and 10). Thienyl nitroalkene also gave good yields with diethyl malonate, acetylacetone and cyclohexanone (Table 3, entries 11–13). Moreover, it is noteworthy that cyclohexenone was also successfully used as the acceptor in this Michael reaction, and it gave a good yield of 75% with acetylacetone (Table 3, entry 14), and a low yield of 32% with diethyl malonate (Table 3, entry 15). Next, we attempted to expand the scope of acceptor to cinnamaldehyde, acrolein and benzalacetone. It was observed that the reaction between cinnamaldehyde and acetylacetone only gave a Knoevenagel product (Table 3, entry 16). Meanwhile, only trace product was detected after 240 h for the reaction of acrolein and cyclohexanone (Table 3, entry 18), but no product was observed for the reaction of acrolein or benzalacetone with ethyl acetoacetate (Table 3, entries 17 and 19).

To our great delight, the Lipozyme TLIM catalyzed Michael reaction showed an enantioselectivity, and this was the first example of enzyme catalyzed asymmetric C–C Michael addition. The reaction between nitrostyrene and ethyl acetoacetate gave 16% ee for the major diastereomer (Table 3, entry 1). The monosubstituted nitrostyrenes only gave low ee values (Table 3, entries 3, 4, and 6–8), but disubstituted nitrostyrene 2,4-dicholo-β-nitrostyrene gave moderate of 52% ee with diethyl malonate (Table 3, entry 5) probably due to the effect of steric hinderence on the induction of the enantioselectivity. Notably, the good enantioselectivity of 83% ee was obtained between thienyl nitroolefin and acetylacetone (Table 3, entry 12). Besides, thienyl nitroolefin gave 43% ee with diethyl malonate (Table 3, entry 11), and 23% ee for the major diastereomer with cyclohexanone (Table 3, entry 13). However, furyl nitroolefin only gave poor enantioselectivities when reacting with diethyl malonate and cyclohexanone (Table 3, entries 9 and 10). Furthermore, cyclohexenone gave 30% ee with acetylacetone (Table 3, entry 14). Finally, when ethyl acetoacetate and cyclohexanone were used as the Michael donors, the products were obtained with moderate to excellent diastereomeric selectivities (dr values of 60:40–98:2) (Table 3, entries 1, 3, 7, 10, and 13).

Finally, we addressed the problem of enzyme recycling. At the end of the reaction of thienyl nitroolefin and acetylacetone, the enzyme was recovered by filtrating from the reaction system, washed twice with ethanol and three times with acetone. The enzyme was dried in the air at r.t., and reused in the next cycle. The reaction was performed four times using same Lipozyme TLIM without adding any new enzyme. The results are shown in Table 4. The gradual decrease in activity and enantioselectivity was observed for the first two cycles (Table 4, cycles 1 and 2). However, the yield and ee dropped evidently at the following cycles (Table 4, cycles 3 and 4). The decrease in yield upon repeated use was probably ascribable to a gradual deposition of tar compounds on the catalyst surface which, after two cycles, began to hamper access of the substrate to the catalytic sites. Furthermore the gradual denaturation of the enzyme may be the other causation. According to the concept proposed by Klibanov and co-workers that the effect of organic solvents on an enzyme is primarily due to interactions with the enzyme-bound, essential layer of water rather than with the enzyme itself [50], we think that the recycle process demolished the prerequisite water layer around the catalyst particles, so it is easy for the enzyme to interact with the organic solvents, causing denaturation.

4. Conclusion

In summary, we describe here the first enzyme catalyzed asymmetric C–C Michael addition using Lipozyme TLIM as a recyclable

biocatalyst. The enantioselectivities up to 83% ee and yields up to 90% were achieved. The reaction conditions including organic solvents, water content, temperature, molar ratio of substrates and enzyme loading were optimized. Lipozyme TLIM can catalyze the biotransformation of a wide-range of substrates in DMSO in the presence of water under mild reaction conditions. The specific catalytic effect of Lipozyme TLIM was demonstrated by the control experiments. This asymmetric Michael addition activity of Lipozyme TLIM provided a novel case of unnatural activities of existing enzymes in organic medium, which is important for discovery of new enzyme activities. Further studies focusing on the improvement of enantioselectivity of this enzyme catalyzed asymmetric transformation are currently under investigation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molcatb.2010.11.011.

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