Lipase-catalysed tandem Knoevenagel condensation and esterification with alcohol cosolvents†

Yi-Feng Lai,[a,b] Hui Zheng,[b] She-Jie Chai,[b] Peng-Fei Zhang*[a,b] and Xin-Zhi Chen*[a]

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Six lipases were screened for their ability to catalyse the Knoevenagel condensation between benzaldehyde and methyl cyanoacetate. Lipase from porcine pancreas tolerated a variety of functional groups on the aromatic ring, produced the highest yields, and also catalysed transesterification of the product in the presence of an alcoholic cosolvent. We show that, in organic solvents, lipase from porcine pancreas has higher activity for this "promiscuous" reaction than for naturally occurring esterification catalysis.

Research in the area of biocatalytic promiscuity has attracted significant attention from chemists and biochemists in recent years.[1] The term "promiscuity" refers to secondary activities of an enzyme in addition to its primary physiological activity. Enzyme promiscuity is hypothesised to contribute to the natural evolution of enzymes and provides new possibilities for exploiting enzymatic synthesis in organic chemistry.[2] The past few years have seen significant advances related to this latent skill of certain enzymes. Berglund reported that wild-type lipases[3] and rationally designed mutants[4] can catalyse the addition of amines, thiols, and β-ketoesters to various Michael acceptors, including acrylonitrile and a,β-unsaturated carbonyl compounds. Additionally, Li *et al.*[5] demonstrated that lipase from porcine pancreas possesses the ability to catalyse asymmetric aldol reactions between ketones and aldehydes. Based on these reports, we conducted experiments to further explore the variety of reactions catalysed by lipases. Here we report that lipase from porcine pancreas is able to catalyse a Knoevenagel condensation and esterification in one pot in the presence of an alcohol cosolvent. Additionally, we found that lipase from porcine pancreas has higher "promiscuous" activity than "natural" activity in organic solvents.

The Knoevenagel condensation is the nucleophilic addition of an active hydrogen compound to an aldehyde or ketone, often followed by dehydration to form a carbon–carbon double bond. Carbon–carbon double bond formation is one of the most useful and fundamental reactions in synthetic organic chemistry—particularly in the synthesis of complex natural products with biological activity. In nature, enzymes catalyse many types of reactions, but carbon–carbon double bond formation generally occurs intramolecularly; for example, the formation of phosphoenolpyruvate is catalysed from 2-phosphoglycerate by hydrolyase. However, it has been reported that some enzymes can catalyse carbon–carbon double bond formation between two substrates in organic media. Hilvert and colleagues reported that oxaloacetate undergoes decarboxylation, followed by aldol condensation with aldehydes, in the presence of macrophomate synthase.[6] Ohta *et al.* demonstrated a decarboxylase-catalysed intramolecular aldol reaction following a decarboxylation.[7] However, large-scale application is restricted by atom economy and the limited industrial outputs of these enzymes. It is interesting, but challenging, to apply the promiscuity of easily acquired porcine pancreatic lipase to these practical syntheses.

We initially began our work by screening a sample of commercially available enzymes for their ability to catalyse the Knoevenagel reaction. All of the enzymes were bought from J&K Chemical Ltd. The reaction of benzaldehyde and methyl cyanoacetate in the presence of enzymes in DMSO at 37 °C was used as a model reaction. Reaction progress was monitored by TLC and GC-MS. As shown in Table 1, porcine pancreatic lipase from porcine pancreas produced the highest yield. Interestingly, this result disagrees with a previous report.[8] In our experiments, no decarboxylative product was measured (0% *versus* 90% in the previous report). We hypothesise that the different results stem from the different specific activity exhibited by the natural structure of different enzymes and from different reaction conditions (they used amine as the additive).[8] In addition, the amylase from hog pancreas also showed high activity, while the denatured enzyme, lipase AY30, bovine serum albumin (BSA) and the control (in the absence of enzyme) demonstrated low activity in this reaction. These results indicate that the enzymatic tertiary structure plays a critical role, excluding the possibility

Table 1 The catalytic activities of the Knoevenagel reaction between benzaldehyde and methyl cyanoacetate[a]

Entry	Catalyst	Yield (%)
1	Lipase (porcine pancreas)	85
2	Lipase A (*Aspergillus niger*)	35
3	Lipase M (*Mucor javanicus*)	40
4	Lipase AK (*Pseudomonas fluorescens*)	4
5	Lipase AY30 (*Candida rugosa*)	2
6	Lipase (recombinant, *Candida Antarctica*)	5
7	BSA	3
8	Amylase (hog pancreas)	74
9	Lipase (denatured, porcine pancreas)	3
10	Control (no enzyme)	3

[a] Reaction conditions: 50 mg enzyme, 1 mmol benzaldehyde, 1 mmol methyl cyanoacetate, 5 mL DMSO, 37 °C, 150 rpm end-over-end rotation for 12 h.

[a]*College of Material Chemistry and Chemical Engineering, Zhejiang University, Hangzhou, 310027, China*
[b]*College of Material Chemistry and Chemical Engineering, Hangzhou Normal University, Hangzhou, 310036, China.*
E-mail: zpf100@163.com; Fax: +86 571 28862867; Tel: +86 571 28862867

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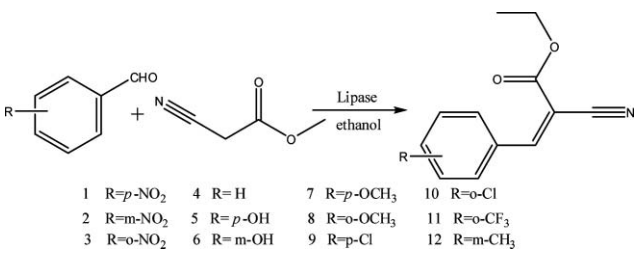
that it simply provides polymeric support. These results were very encouraging and spurred us to further explore this reaction.

Because lipase is known to catalyse the transesterification reaction in organic solvents,[9] we selected ethanol and *tert*-butanol as the substrates (and also solvents) to investigate the possibility of a one-pot Knoevenagel condensation/ester-exchange reaction. A protocol for one enzyme that catalyses two reaction types sequentially would be of interest and, moreover, studying the kinetics of the reaction may help us to better understand the mechanism of the enzyme promiscuity.

Some substituted aromatic aldehydes and methyl cyanoacetate were tested to verify the catalytic effect of the enzyme (Scheme 1). In a typical experiment, an aromatic aldehyde (1 mmol) and methyl cyanoacetate (1 mmol) were added to alcohol (5 mL) containing enzyme (50 mg) and then incubated at 40 °C in an end-over-end rotator at 150 rpm for 12 h. The isolated yield of **1**, **2**, and **3** were all greater than 75%, while the yields of **4** through **12** ranged from 34% to 60%. The yields of aldehydes with electron-withdrawing groups were higher than those with electron-donating groups. The structure of products was confirmed by ^{1}H NMR and MS. Both condensation and transesterification proceeded cleanly in ethanol, and as expected, the transesterification in *tert*-butanol was incomplete (less than 1%) because of steric hindrances. However, in *tert*-butanol, the Knoevenagel condensation proceeded to nearly 100% (GC yield). GC-MS was used to monitor the lipase-catalysed kinetics of the reaction between benzaldehyde and methyl cyanoacetate (Fig. 1). The initial reaction rate of the condensation was 0.54 mM h^{-1}, whereas the rate of transesterification was 0.01 mM h^{-1}; clearly, condensation proceeded much faster than the esterification reaction.



Scheme 1 Knoevenagel condensation combined with esterification in one pot in ethanol.

We also varied temperature, catalyst concentration, and reaction time. We found that the reactions were successful in both aqueous and organic solvents when carried out for 64 h at 60 °C with 1% of the enzyme concentration. When carried out in 1.5% water, the desired product (1) was obtained with a 90% yield by GC.

A generally accepted catalytic mechanism for lipases is that the active site for hydrolysis also contributes to promiscuous catalysis.[10] However; this mechanism cannot explain why some lipases are active while others show no activity under the same reaction conditions. This mechanism also cannot explain our finding that hog pancreatic amylase can also catalyse this reaction in organic solvents (Table 1). Reetz *et al.*[11] recently

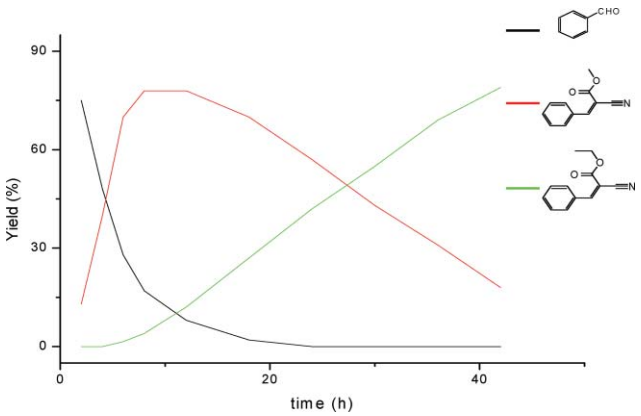


Fig. 1 Kinetic curve of Knoevenagel condensation and esterification of benzaldehyde and methyl cyanoacetate catalysed by lipase in ethanol.

hypothesised that observed promiscuity is not due to the classical mechanism of hydrolytic enzymes, instead suggesting that alternate-site enzyme promiscuity is at work. Although we do not have direct evidence, our results are in line with their conclusions.

In summary, our experiments suggest that several enzymes, especially lipase from porcine pancreas, catalyse the Knoevenagel condensation with esterification with high yields. This is the first time that enzymes had been shown to catalyse two different reactions sequentially to form complex compounds. Although the details of the catalytic mechanism remain unclear, the present observations extend the utility of enzyme promiscuity.

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