Promiscuous zinc-dependent acylase-mediated carbon–carbon bond formation in organic media†

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A zinc-dependent acylase, D-aminoacylase from *Escherichia. Coli*, displays a promiscuous activity to catalyze the carbon–carbon bond formation reaction of 1,3-dicarbonyl compounds to methyl vinyl ketone in organic media.

The seminal work by Klibanov in the early 1980s initiated non-aqueous enzymology.[1] In organic media, many enzymes have demonstrated their activities with non-natural substrates.[2] During the exploration of new activities of enzymes, a growing number of them have been found to be capable of catalyzing not only their "natural" reactions but also one or more alternative reactions.[3] This catalytic promiscuity of enzymes expands largely the application of biocatalysts in synthetic chemistry.[4] Research in this heating area has attracted much attention of chemists and biochemists in recent years.

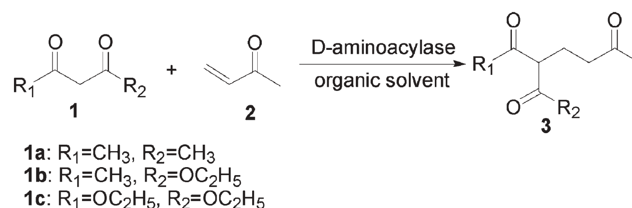
The Michael addition reaction is among the most fundamental types of reactions in organic synthesis. However, there were only rare reports about enzymes which are able to catalyze the Michael addition reactions. Kitazume *et al.* used hydrolytic enzymes to catalyze the Michael-type addition of thiols and amines to triflourinate α,β-unsaturated carbonyl compounds in buffer solution.[5] Our group reported that an alkaline protease from *Bacillus subtilis* showed a remarkable activity to catalyze the Michael addition of N-nucleophiles to acrylates in organic solvents.[6] The wild-type and the Ser105Ala mutant of CAL B could catalyze the Michael-type addition of thiol and amine nucleophiles to a range of α,β-unsaturated carbonyl compounds.[7] However, the relevant report was only limited in the formation of carbon–nitrogen and carbon–sulfur bonds. Recently, Berglund and co-workers reported the unprecedented carbon–carbon bond formation by an engineered mutant of CAL B.[8] This led us to believe that other types of hydrolase could also catalyze this addition reaction to form carbon–carbon bonds.

Here, we surprisingly found that a zinc-binding metallo-acylase, D-aminoacylase from *Escherichia coli*, which naturally catalyzes the hydrolysis of *N*-acyl-D-amino acids, also possesses the promiscuous activity to catalyze the Michael addition of 1,3-dicarbonyl compounds to methyl vinyl ketone. The catalytic specificity of acylases was demonstrated by the combination of different control experiments. According to the control experiments and the observations, a tentative mechanism for the acylase-mediated Michael addition was proposed. This novel Michael addition activity of metallo-acylases is of practical significance in expanding the application of enzymes and in the evolution of new biocatalysts.[9]

We first examined the Michael addition of ethyl acetoacetate (**1b**) and methyl vinyl ketone (**2**) (Scheme 1) in a solventless system. When 6 mmol **1b** was added to a solution containing 10 mg D-aminoacylase and 8.4 mmol **2** at 50 °C for 24 h, a single product was prepared in 35% isolated yield after flash chromatography. The structure of this compound was confirmed by IR, ^{1}H NMR, ^{13}C NMR and MS. In order to improve the activity of enzyme, we carried out the reaction in organic solvent. Some conventional organic solvents with different log *P* values were screened for the enzymatic Michael addition reaction and the results are shown in Table 1. In those solvents such as toluene, chloroform or isopropyl ether, the Michael additions proceeded very slowly and the yields were less than 10% (entries 2–4, Table 1). The result in hexane was almost the same as that in the solvent-free reaction (entries 1, Table 1). In polar solvents such as THF and dioxane, the yields are far from satisfactory (entries 7 and 8, Table 1). D-Aminoacylase showed higher Michael addition activity in some tertiary alcohol solvents (entries 5 and 6, Table 1). The Michael addition in 2-methyl-2-butanol leads to 82% yield in 24 h. Thus, 2-methyl-2-butanol was chosen as solvent in the following experiments.‡

Three types of 1,3-dicarbonyl compounds, ethyl acetoacetate, acetylacetone and diethyl malonate, were tested as nucleophiles for the enzymatic Michael addition reaction in 2-methyl-2-butanol. The progress curves were shown in Fig. 1 and the initial reaction rates were calculated accordingly. The Michael addition of acetylacetone with methyl vinyl ketone proceeded fastest and the initial reaction rate was up to 60.4 mM h^{-1}. The reaction of **1b** also underwent smoothly leading to the corresponding Michael adduct in 82% yield in 24 h. Diethyl malonate exhibited rather low Michael addition activity and only about 1% yield was obtained even after 24 h. This may be attributable to the weaker acidity of α-protons of diethyl malonate or steric hindrance.

Taking into account these results, we performed some control experiments focusing on demonstrating the specific catalytic effect



Scheme 1 Michael addition of 1,3-dicarbonyl compounds to methyl vinyl ketone catalyzed by D-aminoacylase.

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Table 1 The influence of solvents on the enzymatic Michael addition reaction[a]

Entry	Solvent	log *P*	Yield (%)
1	*n*-Hexane	3.9	31.0
2	Toluene	2.6	2.8
3	Chloroform	2.0	1.1
4	Isopropyl ether	1.9	7.0
5	2-Methyl-2-butanol	1.10	82.1
6	2-Methyl-2-propanol	0.79	55.5
7	THF	0.46	17.6
8	Dioxane	−0.5	16.8

[a] *Reaction conditions*: D-aminoacylase 10 mg, ethyl acetoacetate 1 mmol, methyl vinyl ketone 2 mmol, solvent 1 mL, 50 °C for 24 h.

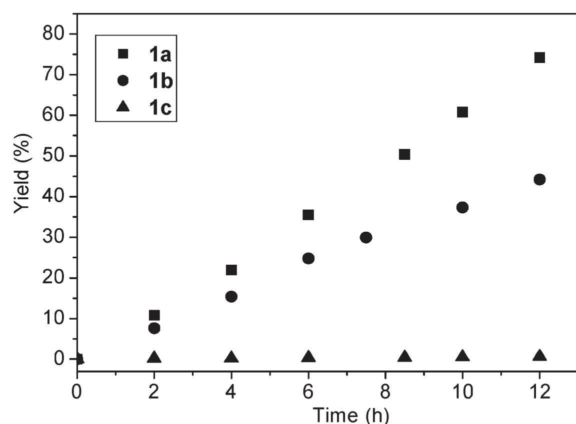


Fig. 1 Progress curve of Michael addition of 1,3-dicarbonyl compounds to methyl vinyl ketone catalyzed by D-aminoacylase in 2-methyl-2-butanol.

of the D-aminoacylase (Table 2). The reaction of acetylacetone with methyl vinyl ketone in the absence of enzyme led to the Michael adduct in very low yield (3.7%) even after 24 h. In contrast, the reactions in the presence of D-aminoacylase was up to 40-fold faster (entries 1 and 2, Table 2). Besides, the initial reaction rate is practically proportional to the enzyme amount, also suggesting the catalytic effect of the enzyme (entries 2 and 3, Table 2). When the reaction was incubated with denatured D-aminoacylase or bovine serum albumin (BSA), both of the initial rates were almost equal to the background reaction (entries 5 and 6, Table 2), ruling out the possibility that the similar amino acid distribution on the protein surface has promoted the process.

Table 2 Initial rates (V_o) of the Michael addition between acetylacetone (1 M) and methyl vinyl ketone (2 M)

Entry	Catalyst	Amount/mg	V_0/mM h^{-1}	V_r[a]
1	—	—	1.5	1.0
2	D-Aminoacylase	10	60.4	40.3
3	D-Aminoacylase	5	30.8	20.5
4	Acylase "Amano"	10	2.2	1.5
5	BSA	10	2.7	1.8
6	denatured[b]	10	2.3	1.5
7	CAL B	10	6.2	4.1
8	Amano Lipase M	10	7.2	4.8
9	CCL	10	2.1	1.4

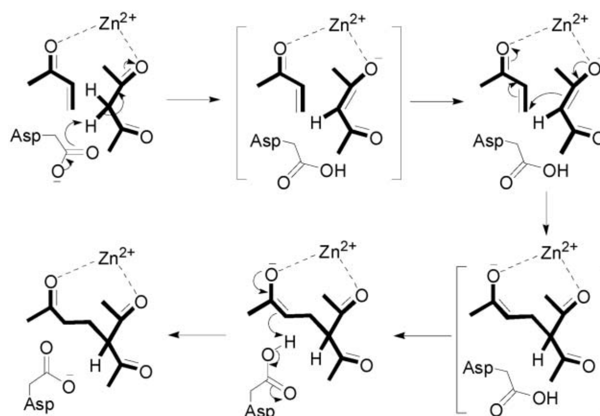
[a] Relative initial rate to the reaction in absence of enzyme. [b] Pretreated with urea at 100 °C for 6 h.

Acylase "Amano" from *Aspergillus oryzae*, which is also a zinc-dependent acylases, did not show any Michael addition activity. Three widely used hydrolases, Lipase from *Candida cylindracea* (CCL), CAL B and Amano lipase M, can not accelerate the reaction efficiently in 2-methyl-2-butanol either (entries 7–9, Table 2). All these results suggest that the tertiary structure and the specific spatial conformation of D-aminoacylase are responsible for the Michael addition reaction of 1,3-dicarbonyl compounds to methyl vinyl ketone.

It was also observed that the D-aminoacylase-mediated Michael addition reaction was strongly affected by the order of adding reagents. When acetylacetone was added to the solutions containing enzyme and methyl vinyl ketone, the reaction proceeded rapidly and the initial reaction rate was about 60.0 mM h^{-1}. However, only slow reaction rate was observed when methyl vinyl ketone was added to the solution of enzyme and acetylacetone; its initial reaction rate was only about 20.0 mM h^{-1}. It is worthwhile to note that D-aminoacylase could also catalyze the Michael addition of acetylacetone and 2-cyclohexene-1-one, although the reaction rate was much slower.

The proposed mechanism in Scheme 2 might explain the observed addition order activity. The generally accepted zinc-dependent acylase mechanism usually involves the polarization of a carbonyl group and nucleophile water by the zinc ion in the active sites. A highly conserved Asp plays a key role in the proton transfer from the nucleophile water to the leaving group.[10] The proposed mechanism would start with the accommodation of addition acceptor (methyl vinyl ketone) and nucleophile (acetylacetone) in the active site. The interaction of the tightly bound zinc ion with the carbonyl group of methyl vinyl ketone would increase the electrophilic ability of Michael acceptor. On the other hand, one of the carbonyl groups of acetylacetone would coordinate with the zinc ion, thus rendering the acetylacetone more nucleophilic. Then, Asp366 deprived the C3-H of acetylacetone and the nucleophile would add simultaneously to the β-carbon of methyl vinyl ketone to form the enolate intermediate, which could be stabilized by the zinc ion in the active site. Finally, the Asp, now functioning as a general acid, would deliver the proton to complete the reaction.

This tentative mechanism was based on the proposal that the zinc ion in the active site coordinated with methyl vinyl ketone and



Scheme 2 Proposed mechanism of D-aminoacylase-catalyzed Michael addition.

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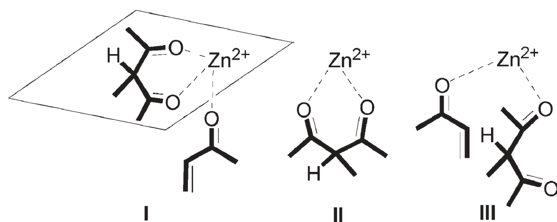

Scheme 3 Possible interactions of reactants with zinc ion in the active site of enzyme.

one of the carbonyl oxygens of acetylacetone. In fact, the interactions of zinc ion in the active site with reactants can occur in three possible ways as shown in Scheme 3. If both reactants coordinated simultaneously to the zinc ion in the active site, the Michael acceptor should adopt a *s-cis* conformation as shown in **I**. However, the fact that D-aminoacylase could catalyze the Michael addition of acetylacetone and 2-cyclohexene-1- excluded automatically the possibility of **I**. On the other hand, because the zinc ion in the active site have already tightly bound with Cys96, His220 and His250,[10] the three carbonyl oxygens of acetylacetone and methyl vinyl ketone could not simultaneously coordinate to the zinc ion in the active site. Therefore, only two carbonyl oxygens of the reactants could coordinate with the zinc ion in the active site to form **II** or **III**. The experiment results of different orders of adding reagents are inclined to support the formation of **III**. Thus the proposed mechanism was based on the formation of **III**.

In conclusion, a facile biotransformation path to perform Michael additions between 1,3-dicarbonyl compounds to methyl vinyl ketone has been developed by utilizing promiscuous D-aminoacylase as biocatalyst. The catalytic promiscuity of D-aminoacylase was demonstrated by the combination of different control experiments. Based on the experiments, a tentative mechanism was proposed.

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Notes and references

‡ *Typical experimental procedure*: In a conical flask containing 10 mg D-aminoacylase, a solution of internal standard (dodecane) and methyl vinyl ketone (2 mmol) in 1 mL 2-methyl-2-butanol was incubated at 50 °C and 250 rpm (orbitally shaken) for 20 min. Then, 1,3-dicarbonyl compound (1 mmol) was added in order to initiate the reaction. Samples were withdrawn from the reaction and directly analysed using a GC (SE-54 capillary column, FID detection; oven temperature: from 60 to 200 °C, rate of heating 20 °C min.$^{-1}$). All the compounds were spectroscopically characterized (IR, ^{1}H, ^{13}C NMR and MS) and analytically compared (GC) with authentic samples prepared by conventional methods.

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