

Biocatalysis

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Carboxylic Acid Reductase Can Catalyze Ester Synthesis in Aqueous Environments

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Abstract: Most of the well-known enzymes catalyzing esterification require the minimization of water or activated substrates for activity. This work reports a new reaction catalyzed by carboxylic acid reductase (CAR), an enzyme known to transform a broad spectrum of carboxylic acids into aldehydes, with the use of ATP, Mg²⁺, and NADPH as co-substrates. When NADPH was replaced by a nucleophilic alcohol, CAR from *Mycobacterium marinum* can catalyze esterification under aqueous conditions at room temperature. Addition of imidazole, especially at pH 10.0, significantly enhanced ester production. In comparison to other esterification enzymes such as acyltransferase and lipase, CAR gave higher esterification yields in direct esterification under aqueous conditions. The scalability of CAR catalyzed esterification was demonstrated for the synthesis of cinoxate, an active ingredient in sunscreen. The CAR esterification offers a new method for green esterification under high water content conditions.

Esters are economically important chemicals because of their wide applications as fragrances, flavor agents, cosmetics, soaps, medicines, and herbicides/pesticides.^[1] Esters can be prepared chemically by reacting a carboxylic acid with an alcohol in the presence of an acid or base catalyst, such as in the Fischer^[2] and Steglich^[3] esterifications. Although existing catalytic technologies employed in esterification are efficient, these reactions are not environmentally friendly because they require the use of organic solvents, non-biodegradable catalysts, and elevated temperatures. Recently, the ester synthesis capability of acyltransferase from *Mycobacterium smegmatis* (MsAcT) in water was reported. However, it was shown to require a pre-activated acid for transesterification.^[4] As biocatalysis by enzymes or metabolically engineered cells

are generally accepted as sustainable means for production of fine chemicals in the future, the identification of new enzymatic reactions to catalyze esterification in aqueous environments will pave the way for performing esterification with metabolically engineered cells.^[5] Up until now, reports of efficient enzymatic esterification reactions in aqueous environments are limited.^[6] For examples, although non-ribosomal peptide synthetase (NRPS) can catalyze esterification and amidation, the use of NRPS as a biocatalyst is challenging, as NRPS reactions are substrate-specific and require a defined reaction order, thus, limiting the range of convertible carboxylic acids.^[7] CoA-dependent acyltransferases and acetyltransferases are also known to play a role in lipid biosynthesis. Despite their capability to synthesize ester, the reported enzymes are mostly membrane-bound and their substrate scopes are specific towards fatty acyl CoAs or acetyl CoA.^[8]

CAR catalyzes the reduction of carboxylic acids to aldehydes.^[9] It is a useful enzyme for the synthesis of many high-value compounds.^[9b,10] The structure and mechanism of CAR are similar to those of NRPS.^[9b,11,12] In the first catalytic step, ATP is used for the adenylation of the carboxylic acid to form the acyl-AMP intermediate. Through thiolation, the acyl-AMP intermediate is attached to the phosphopantetheine (PCP) group of CAR. This PCP moiety then transfers the thioester intermediate to the reductase domain, where NADPH is used in the reduction, liberating the aldehyde product from the protein.^[9b,11c,12] Flitsch's group demonstrated that CAR can catalyze amidation instead of reduction with substitution of an amine.^[13] The reaction mechanism of carboxylic acid amidation by CAR is thought to be similar to the mechanism of NRPS.^[14]

Inspired by the work mentioned above, we aimed to explore whether CAR can catalyze esterification in water, apart from to carboxylic acid reduction and amidation, for future application in metabolically engineered cells. Previously, CAR has also been shown to be involved in the chemo-enzymatic synthesis of esters by reducing carboxylic acids into aldehyde substrates for the subsequent Wittig reaction to generate α,β -unsaturated ester products.^[15] However, the ability of CAR alone to catalyze esterification has not been reported.

We first carried out the standard CAR reaction with benzoic acid as the substrate in methanol. CAR used in our experiments was of the highest purity (> 99%; Supporting Information, Figure S1) to avoid promiscuous activities from other contaminating enzymes. Apart from the formation of the benzaldehyde product, a side product, identified as methyl benzoate was also detected. This discovery led us to

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further explore the condensation between carboxylic acids and alcohols catalyzed by CAR. When NADPH was omitted from the reaction, more methyl benzoate product was observed, but was still only found in trace amounts (data not shown). Therefore, the conditions were adjusted to maximize the ester production by CAR. The dependence on pH was evaluated by initially varying the pH of the buffer system. The preliminary esterification was conducted at 37 °C and consisted of 0.78 μM *Mycobacterium marinum* CAR (*MmCAR*), 1 mM benzoic acid, 4% methanol, 5 mM of ATP, and 10 mM of MgCl_2 at their final concentrations in either 100 mM Tris-HCl or 100 mM Na_2CO_3 buffer at pH 7.5–10.5. Methyl benzoate was detected using GC-MS and the yields were calculated from a calibration curve based on methyl benzoate standards. The highest yield of methyl benzoate, 0.59%, was obtained in the 100 mM Na_2CO_3 buffer system at a pH of 10.0 (Supporting Information, Table S1). Because the expression level in bacteria of the apo form of *MmCAR* was higher than that of the holo form, which requires co-expression of the Sfp protein to attach a PCP group, and because both forms of CAR gave similar yields of methyl benzoate product (results not shown), all subsequent experiments were performed using only apo *MmCAR*. Although, the apo *MmCAR* prepared may be a mixture of apo or holo forms due to plausible modification by *E. coli*, this issue does not affect CAR-catalyzed esterification because PCP is not involved in the esterification mechanism (see later).

We then further explored the effects of additives which might be mechanistically advantageous for enhancing the esterification. According to the mechanism of CAR, the first step of the reaction is the formation of acyl-AMP and acyl-PCP to activate the carbonyl groups such that they are more electrophilic than in the carboxylic acid form, making them more susceptible to nucleophilic attack by a hydride from NADPH. Most chemical esterification reactions also require activation of the carboxylic acid to promote nucleophilic addition. Various additives that could help activate the carboxylic acid were supplemented into the 100 mM Na_2CO_3 pH 10.0 buffer. All reactions were monitored for the conversion of cinnamic acid to methyl cinnamate (Supporting Information, Table S2).

The first group of additives used were thiol containing compounds, to see whether any PCP mimics would enhance ester production (Supporting Information, Table S2, entries 1–3). The other groups of additives were chosen based on the basic principles of chemical catalysis.^[16] Guanidine and imidazole can mimic functions of general acid and base catalysts commonly found in enzymes.^[16c] The bifunctional 2-hydroxypyridine can act as a concerted acid and base catalyst,^[16c] which can also help generate a carboxylate anion. Thiamine^[16b] and imidazole^[16c] can serve as nucleophilic catalysts. Enhancement effects from imidazole should be the most promising, because histidine serves as a general acid/base catalyst (catalytic residue) in many enzymes.^[17]

Indeed, the results showed that among the seven additives, imidazole at the concentration of 100 mM was the most effective compound for improving the production of methyl cinnamate (Supporting Information, Table S2, entry 7). Since imidazole significantly enhanced the production of methyl

cinnamate, it was evaluated as a standalone buffer. The optimum concentration of imidazole as a standalone buffer was 200 mM (Supporting Information, Figures S3 and S4). When CAR was not included in the 200 mM imidazole buffer system, no methyl cinnamate peak was detected in the HPLC-DAD chromatogram. Next, the effect of temperature on the production of methyl cinnamate was examined in the original 100 mM Na_2CO_3 and in the new 200 mM imidazole buffer systems. CAR worked most efficiently at a mild temperature such as at 25 °C (Supporting Information, Figure S5). The effect of alcohol was also evaluated for the production of other esters (Supporting Information, Figures S6 and S7). Methanol, at a concentration higher than 5% decreased the yield of methyl cinnamate, possibly due to the inhibitory effects on CAR (Supporting Information, Figure S7). Details of product identifications are shown in the Supporting Information, Figures S8–S13.

We investigated the mechanistic role of imidazole in enhancing CAR-catalyzed esterification (Supporting Information, Figure S14–S19). We hypothesized that imidazole either reacts with the acyl-AMP intermediate to form acyl-imidazole which may somehow speed up esterification or serve as an acid/base catalyst for the esterification. We found that that CAR cannot convert cinnamoyl-imidazole and alcohol into an ester in the absence of imidazole, eliminating the possibility that acyl-imidazole is an intermediate in the CAR-catalyzed esterification. The data suggest that imidazole likely serves as a general acid/base catalyst to mediate proton transfer during esterification (Figure 1).

To elucidate the involvement of the reductase domain and PCP group in CAR-catalyzed esterification, we compared esterification of methyl cinnamate using apoCAR, the truncated CAR in which the reductase domain was removed, and the S685A variants of apoCAR and truncated CAR in which PCP cannot be attached.^[12] Results (Supporting Information, Figure S20) showed that all enzymes exhibited similar activities, indicating that the reductase domain and the PCP group is not involved in esterification (Figure 1).

With the optimized conditions, steady-state kinetics parameters of CAR-catalyzed esterification of acetic acid, cinnamic acid, and benzoic acid were investigated. Among these substrates, CAR was most efficient at converting

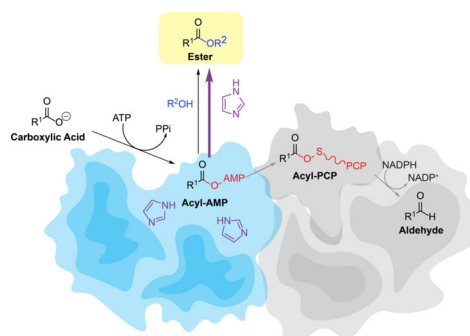


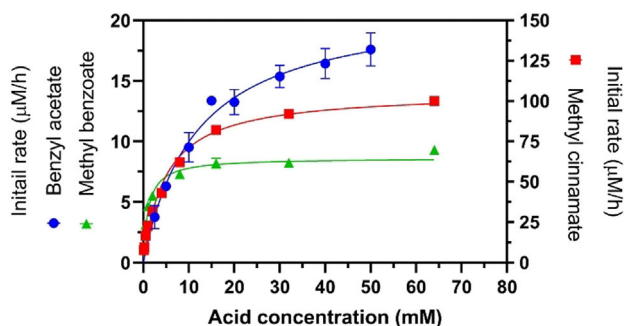
Figure 1. The proposed mechanism of apo CAR esterification enhanced by imidazole additive. R^1 = alkyl (C_5H_{11} , C_6H_{13} , C_7H_{15} , C_9H_{19}), Ph; R^2 = alkyl (CH_3 , C_4H_9 , C_6H_{13} , C_8H_{17} , C_9H_{19}), Bn (Supporting Information, Table S3).

cinnamic acid to methyl cinnamate, with the highest turnover number of 21.2 h⁻¹ (Figure 2; Supporting Information, Figures S21–S27).

To explore substrate scopes and preference in reaction conditions of CAR in comparison to other enzymes known to catalyze reactions in aqueous environments, we explored

esterification by CAR versus lipase (Novozyme 435)^[17b,18] and MsAcT under similar conditions (Supporting Information, Figures S28–36, Tables S4–S5). As these enzymes operate via different reaction mechanisms, their requirement of substrate scopes and reaction conditions would be different.

While lipase performed best in the neat condition with minimal water content (yielded up to 62.5 % methyl cinnamate, Supporting Information, Figure S33), it could not catalyze efficient esterification under aqueous environment (0–2 % of product formation). CAR could produce decent yields (41 % methyl cinnamate) of these ester products, as shown in Table 1, which included the anti-inflammatory agent (methyl ferulate),^[19] an ester with tobacco mosaic virus inhibitory activity (methyl 4-hydroxyphenylacetate),^[20] a halogen containing ester (methyl 2,4-dichlorophenoxyacetate), and the FDA approved sunscreen active ingredient (cinoxate)^[21] (Table 1; Supporting Information, Figure S28). CAR can also use a variety of alcohols (Supporting Information, Figure 29). Cinoxate could also be produced with an isolated yield of 32 % from the scaled-up synthesis by CAR (Table 1; Supporting Information, Figures S30–S32). CAR esterification was also tested at a milder pH of 7.5 and it outperformed lipase in all reactions for the production of various flavor and fragrance esters in aqueous solutions (Supporting Information, Tables S4, S5).



Substrates	Kinetic parameters		
	k_{cat} (h ⁻¹)	K_m (mM)	k_{cat}/K_m (mM ⁻¹ h ⁻¹)
Acetic acid ^[a]	1.08 ± 0.08	11.54 ± 3.05	0.09
Cinnamic acid ^[b]	21.18 ± 0.23	4.86 ± 0.12	4.36
Benzoic acid ^[c]	1.73 ± 0.03	1.08 ± 0.05	1.60

Figure 2. Steady-state kinetic parameters of CAR for the substrates: acetic acid, cinnamic acid, and benzoic acid. CAR concentrations used were 20 μM for acetic acid and 5 μM for other acids.

Table 1: CAR in the synthesis of esters.

$\text{R}^1\text{COOH} + \text{HO-R}^2 \xrightarrow[\text{Imidazole buffer}]{\text{CAR, Mg}^{2+}, \text{ATP}} \text{R}^1\text{COOR}^2$				
Acid	Alcohol	Ester	Yield [%] ^[a]	CAR ^[c]
	HO-		19 ^[d]	8
	HO-		43 ^[d]	46
	HO-		13 ^[d]	14
	HOCH ₂ CH ₂ OC ₂ H ₅		10 ^[d] (32) ^[e]	4
	HO-		41 ^[f]	ND
	HO-		3.5 ^[g]	ND
	HOCH ₂ Ph		5.6 ^[h]	ND

Reaction contained 1 mM acid, 5 mM ATP, 10 mM MgCl₂, CAR, alcohol, and 200 mM imidazole buffer. [a] Yield determined by GC-MS after shaking at 60 rpm, 25 °C. [b] Reaction perform in imidazole buffer pH 10.0. [c] Reaction perform in imidazole buffer pH 7.5 [d] 50 μM CAR, 123.5 mM MeOH or 51.6 mM 2-ethoxyethanol, 24 h. [e] Isolated yield after 45 h from a 20 mL reaction scale. [f] 10 μM CAR, 1.24 M MeOH, 24 h. [g] 5 μM CAR, 1.24 M MeOH, 8 h. [h] 20 μM CAR, 96.54 mM benzyl alcohol, 4 h. ND = not determined.

We then further compared the esterification reaction of CAR with that of MsAcT (Supporting Information, Figures S34–S36). MsAcT efficiently catalyzes transesterification of vinyl acetate with benzyl alcohol (a yield of greater than 50% within 1 hour, data from the Supporting Information, Figure S35). However, its ability to perform direct esterification from acetic acid is limited; the yield reached the highest level (ca. 25%) after the first hour and decreased to be less than 20% after 24 hours. This observation suggests that MsAcT catalyzes the reverse hydrolysis reaction as well as esterification. For direct esterification of acetic acid and benzyl alcohol, CAR could not catalyze direct esterification well either (yield of 5.6%, Table 1). However, CAR could catalyze much better esterification of bulkier acids with the product yield up to 43% (Table 1 and Figure 2) while direct esterification of these bulkier acids was significantly impaired (<1% yield) in the reaction by MsAcT (Supporting Information, Table S5).

These results demonstrated that, in aqueous conditions, CAR had a clear advantage over lipase and MsAcT in direct esterification, especially with bulky aromatic acid as substrates. Esterification of some cinnamic acid derivatives, by Novozyme 435 requires high temperatures^[22] and the minimization of the amount of water to give high percentages of conversion,^[22d] while MsAcT requires an activated ester as a substrate. Esterification by CAR is advantageous in that it is thermodynamically irreversible, as the presence of water does not result in the reverse reaction of converting the product back to the acid form. A plausible explanation could be that CAR does not contain a catalytic triad like lipase or other transferase enzymes, where the amino acids of the catalytic triad can also promote the nucleophilic attack of water.^[15] However, CAR has the disadvantage of using costly ATP as a co-substrate which requires further process optimization in the future, possibly using an ATP regenerating system to convert AMP back to ATP by a low cost polyphosphate.^[23]

In comparison to the production of amides previously reported,^[13] without adjustment of reaction buffer to imidazole, esterification behaves poorly (less than 1% conversion of methyl benzoate compared to 11% conversion of benzamide). However, with imidazole enhancement, methyl cinnamate was produced up to 41% in 24 hours (Table 1) compared to 22% conversion of cinnamamide at 24 hours. An improved esterification exhibits comparable efficiency to that of amidation. However, the highest percentage conversion of amide synthesis (71% conversion to ilepcimide at 24 hours) is still higher than any tested esterification condition (46% yield of methyl 4-hydroxyphenylacetate in the Supporting Information, Table S5).

In conclusion, carboxylic acid reductase enabled the transformation of carboxylic acids into esters under mild aqueous conditions at 25 °C with a broad substrate range for carboxylic acids and alcohols. This new activity of CAR might even allow it to be used in a whole cell biocatalyst or in metabolic engineering applications for the synthesis of esters, as it also worked well at pH 7.5.

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Conflict of interest

The authors declare no conflict of interest.

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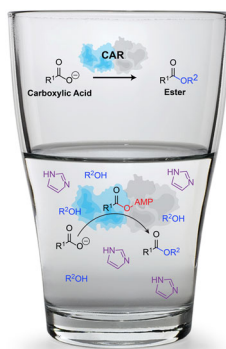
Communications



Biocatalysis

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Carboxylic Acid Reductase Can Catalyze
Ester Synthesis in Aqueous Environments



An efficient and green method is presented for the synthesis of esters using carboxylic acid reductase as a biocatalyst under mild aqueous conditions.