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The unexpected kinetic effect of enzyme mixture: The case of enzymatic esterification

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ABSTRACT

During the studies towards synthesis of carboxylic acids esters, using ethyl carbonate and carboxylic acid as substrates, we found that different single enzyme systems provide model ethyl 3-phenylpropanoate in very low yield. Systematic studies proved that combination of two or more enzymes enhances yield of reaction. Application of selected enzyme mixture for enzymatic esterification of various carboxylic acids provided respective esters in excellent yields. Unexpectedly, the same reaction performed with mixture of five enzymes proceeds in almost quantitative yield. For racemic substrates reaction catalyzed by enzyme mixture was found to be enantioselective. To the best of our knowledge it is the very first evidence of cooperation between multiple enzymes in organic solvents.

1. Introduction

Esterification is one of the most fundamental reactions in organic chemistry [1]. Esters are used as fragrances, resins, plasticizers, bulk and fine chemicals in industry. Classic Fischer esterification requires heating a carboxylic acid and alcohol with an addition of acid catalyst. It is energy consuming and fully reversible process, in which water must be removed from reaction mixture. Additionally this reaction usually requires high amount of catalyst and excess of reagents and it generates a lot of waste, which is incompatible with rules of green chemistry [2]. During the last few years new, environmentally friendly and efficient catalysts and conditions for esterification have been established, however the undesirable presence of heavy metals or other inorganic compounds frequently is found in products [1]. Nowadays the increasing demands for clean processes have led to the development of biodegradable catalysts and mild conditions [2]. Typical catalysts for this reaction are mineral acids or inorganic compounds *e.g.* titanium salts [3]. Of late a great number of papers about modifications of standard esterification procedure were published. Ram and Palaniappan used polyaniline salts to direct esterification of carboxylic acids with alcohols [4], however those experiments require high temperature. Sobkowicz et al. provided esterification of carbon and cellulosic nanoparticles, also in high temperature and with the addition of hydrochloric acid [5]. Catalysis by lewis acids was modified by Yamamoto and co-workers [6]. They tapped synergistic catalytic effect in Zr(IV) and Fe(III), Ga(III) or Sn(IV) binary metal complexes to catalyze condensation between acid and alcohol. They obtained products in high yields, however conditions of the reaction were harsh.

For the synthesis of esters to be used in pharmaceutical or food industry special procedures are required that provide products without any traces of inorganic and organic impurities with special attention has been paid to enzymatic procedures. Enzymes are specific, efficient and biodegradable biocatalysts [7a]. Moreover they are very substrate selective, which in some instances is a plus, but in many others unfortunately diminishes their application. Activity of enzymes could be modulated with organic cosolvents [7b], immobilization [7c], microwave irradiation [7d]. Esterification catalyzed by enzymes is reversible reaction, and some modifications were performed to make this process irreversible. For example, the molecular sieves are added to absorb the water produced upon reaction, however this approach is problematic in large scale syntheses [7a]. Another way to overcome the reversibility of esterification is performing the reaction with specific alkoxy or acyl-donors [8–10]. Recently, interesting example of irreversible esterification of carboxylic acids using carbonates as alkoxy-donors was reported. The enzymatic process was described for esterification of fatty acids with lipase from *Mucor miehei*, however the products were obtained in low yields [11]. Carbonates are readily available and belongs to relatively non-toxic reagents, which are used as substrates or solvents. Naproxen enzymatic synthesis [12a] along with preparation of some polyunsaturated fatty acids (PUFA)

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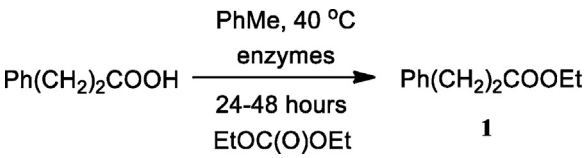

Scheme 1. Model reaction of phenylpropanoic acid with diethyl carbonate.

esters [12b] with the use of carbonates, has been recently reported. However, due to very high substrate selectivity of this particular enzyme, the esterification reactions with carbonates are still limited. Thus, modulation of enzymatic substrate selectivity is very important factor.

In our studies we attempted to broaden substrate acceptability in irreversible enzymatic esterification methods. Our method provides compounds, which are commonly used in food and pharmaceutical industries [13]. Studies on enzymatic reaction of 3-phenylpropanoic acid and other carboxylic acids with carbonates are presented, which led to discovery of unique, multi-enzymatic system for irreversible esterification of diverse carboxylic acids.

2. Results and discussion

Initial experiments were focused on irreversible, esterification method providing esters of carboxylic acids without inorganic impurities, applying methodology utilizing dialkyl carbonates as alkoxy group donors. For synthesis of 3-phenylpropanoic acid ethyl ester diethyl carbonate was used according to the methodology described by Morrone et al. [12]. In this reaction course diethyl carbonate serves as alkoxy group donor providing respective ester. However, there is a need of alcohol additive to begin reaction course [12]. We proposed a different approach to esterification of carboxylic acids with dialkyl carbonates exploiting multienzymatic catalyst.

2.1. Effect of various biocatalysts

Screening of biocatalysts was performed using over 45 commercially available enzymes in the model reaction conducted in toluene at 40 °C (Scheme 1).

In order to speed up screening process 45 enzymes were divided into nine reaction mixtures (vials). Each group consisted of five different enzymes. It was observed that only one group consisted of: lipase from wheat germ, acylase I from *Aspergillus melleus*, papain crude powder, Amano lipase AK from *Pseudomonas fluorescens*, and Novozym 435 catalyzed model reaction efficiently. Results are presented in Table 1.

Preparative scale reaction provided ester **1** (model compound) with above mentioned enzyme mixture in 96% isolated yield (Table 1, entry 1). In other groups formation of ester **1** was not observed. To determine which of enzymes is responsible for the formation of ester **1**, the series of experiments with single enzyme from the active group were performed: lipase from wheat germ, acylase I from *A. melleus*, papain crude powder, Amano lipase AK from *P. fluorescens*, and Novozym 435 (Table 1, entries 2–6). Surprisingly in all reactions with a single enzyme, no significant formation of **1** was observed (Table 1, entries 1–5). This somehow confusing observation led to the conclusion that two or more enzymes are responsible for observed catalytic effect. To identify the catalytic effect of enzymes pairs, second set of experiments was performed (Table 1, entries 7–16). According to the data included in Table 1 under standard conditions product **1** was obtained for some of these two-enzyme mixtures (Table 1, entries 9, 10, 13–16). Only one combination of two enzymes gave the product in substantially higher yield 14% (Table 1, entry 14). However, this yield was still much lower in respect to the yield obtained with five-enzyme mixture. Therefore next set of experiments was performed with mixture of three different enzymes (Table 1, entries 17–26). Only one combination of enzymes successfully provided product in 50% yield (Table 1, entry 19). Two other enzyme mixture provided product in 44% and 42% (Table 1, entries 24 and 25). The presence of two enzymes: Novozym 435 and acylase I from *A. melleus* was found to be crucial for yield enhancement. The same phenomenon was observed in reactions catalyzed by mixture of four enzymes (Table 1, entries 27–31) and the highest yield of reaction was 78% (Table 1, entry 30). It is interesting to note that the absence of Novozym 435 in enzyme mixture substantially decreased the yield of reaction (Table 1, entry 26). This observation indicates that Novozym 435 is crucial for the reaction. However, reaction catalyzed by initial five-enzyme mixture provided product in substantially higher yield (Table 1, entry 1). The course of reaction was also studied by GC method. It turned out that only small amount of ethanol (less than 1%) was present in reaction mixture with carboxylic acid. Without carboxylic acid formation of ethanol was not observed, what is in opposition to mechanism proposed previously [12a]. In order to get inside into catalytic effect of lipase from *Candida antarctica* present in Novozym 435, thermally deactivated Novozym 435 was used together with four other enzymes. In this experiment only traces of product 1 were obtained (Table 1, entry 32). Changing deactivated Novozym 435 to native lipase from *C. antarctica* in enzyme mixture the same result was obtained (Table 1, entry 33). However, when reaction was conducted with those two biocatalysts together with four other enzymes, the product was obtained in 10% yield (Table 1, entry

Table 1
Esterification of 3-phenylpropanoic acid with combinations of enzymes.

Entry	Enzyme	Yield [%]
1	A, B, C, D, E	96
2	A	<1
3	B	<1
4	C	<1
5	D	<1
6	E	<1
7	A, B	<1
8	C, D	<1
9	B, C	5
10	B, D	5
11	A, C	<1
12	A, D	<1
13	A, E	5
14	B, E	14
15	C, E	5
16	D, E	5
17	A, B, C	<1
18	A, B, D	<1
19	A, B, E	50
20	A, C, D	<1
21	A, C, E	13
22	A, D, E	10
23	B, C, D	<1
24	B, C, E	44
25	B, D, E	42
26	C, D, E	5
27	A, B, C, D	<1
28	A, B, C, E	38
29	A, B, D, E	66
30	A, C, D, E	78
31	B, C, D, E	66
32	A, B, C, D, F	<1
33	A, B, C, D, G	<1
34	A, B, C, D, F, G	10
35	F, G	<1

[A] papaine, [B] acylase I from *Aspergillus melleus*, [C] lipase from wheat germ, [D] Amano lipase AK from *Pseudomonas fluorescens*, [E] Novozym 435, [F] deactivated Novozym 435, [G] lipase from *Candida antarctica*.