

Enzymatic Esterification of Carboxylic Acids and Higher Alcohols in Organic Medium

D.T. Mirzarakhmetova

Abstract—The studying of enzymatic esterification of carboxylic acids and higher alcohols was performed by esterase *Saccharomyces cerevisiae* in water-organic medium. Investigation of the enzyme specificity to acetic substrates showed the best result with acetic acid in esterification reactions with ethanol whereas within other carboxylic acids the esterification decreased with acids: hexanoic > pentanoic > butyric > decanoic. In relation to higher alcohols C₃-C₅, esterification increased with alcohols propanol < butanol < amylo. Also it was determined that esterase was more specific to alcohols with branched chain such as isobutyl alcohol and isoamyl alcohol. Data obtained may have important practical implications, for example, for application of yeast esterase in producing various volatile esters as well as in enzymatic transformation of volatile acids and toxic fusel alcohols into volatile esters by providing the production of the high quality alcoholic beverages with reduced content of higher alcohols as well as with improved degustational and hygienic properties.

Keywords—enzymes in non-conventional media, esterification, higher alcohols, volatile esters, yeast esterase

I. INTRODUCTION

ESTERASES usually catalyze hydrolysis of esters in watery medium. However, there is evidence that in organic media they are also capable of actively catalyzing the binding of certain alcohols into esters by virtue of their ester forming function [1]. They do not exhibit esterification activity in the presence of low concentrations of organic phase, because the equilibrium is shifted towards the hydrolytic reaction. Although water-organic media cause the majority of enzymes to lose their activity, there are several examples of enzyme activation, rather than inactivation, at high concentrations of organic material in the medium. For instance, cutinase of *Fusarium solani pisi* synthesizes ethyl esters of medium-chain fatty acids [2], lipase forms ethyl esters (R,S) of hydroxyphenylacetate and geranyl propionate [3] as well as betulinic acid ester [4] in a water free medium. The biochemistry of such reactions has not been studied in detail, e.g., in esterase-organic phase model system. Preliminary evidence suggests that the use of fatty acids and higher alcohols in this system is appropriate [5], [6]. In this work, we aimed to study the substrate specificity of intact esterase *Saccharomyces cerevisiae* in a water-organic (acetonitrile) medium containing fatty acids (C₄-C₁₀) and higher alcohols (C₃-C₅) and determined the enzyme specificity toward acidic and alcoholic substrates within the esterification.

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II. EXPERIMENTAL METHOD

A. Materials

The intact esterase *Saccharomyces cerevisiae* has obtained from the Institute of Microbiology, Academy of Sciences (Tashkent, Uzbekistan). Acetonitrile, ethanol, acetic acid, hexanoic acid, propanol, isopropanol, isobutanol, amylo, isoamylo were from Sigma-Aldrich (Moscow, Russia). Butanol, octanoic acid and decanoic acid were from Sigma (Moscow, Russia). Amyl alcohol, butyric acid, pentanoic acid (valeric acid) were from Aldrich (Moscow, Russia).

B. Methods

The characteristics of the enzyme in water-organic medium were studied using a 20% acetonitrile in 0.1 M phosphate buffer, pH 8,0; fatty acids (C₄-C₁₀) and alcohols (C₂-C₅) served as the substrate. The esterification activity of esterase was determined by methods described in [7]. One unit of esterification activity of esterase corresponded to the conversion of 1 μM isoamyl alcohol into ester per 1 hour under optimal conditions (30 °C, pH 8, 48 h). Alcohols and esters quantification was carried out with an AT 6890 (Agilent Technologies, USA) gas chromatograph in combination with a flame ionization detector (an HP-FFAP capillary column, 50 m length, 0,32 mm in inner diameter, 50 μm film, hydrogen and air consumption were 30 and 300 mL, respectively) according to the State Standard [8]. Chromatographic separation was carried out under thermostat temperature conditions from 77 to 160 °C, the rate of carrier gas (nitrogen) was 1,2 mL/min and flow division mode was 1:30. The devise control and results processing was provided by ChemStation software. Quantitative analysis of proteins was performed according to Lowry method [9].

III. RESULTS AND DISCUSSION

In order to check for the ability of esterase to convert carboxylic acids into esters in organic media, a series of experiments related to the determination of enzyme specificity were performed. The reaction of blend containing 20% organic phase (acetonitrile) and ethanol (0.2 mM) and carboxylic acids with the carbon chain C₁-C₁₀, which were treated with esterase under conditions favoring optimum conversion (30 °C, pH 8,0) [7]. Determination of the esterase activity from initial substrate concentration was carried out with acid concentrations ranging from 0.2 to 0.5 mM in 1 mL incubation mixture. As presented in Fig.1, esterase showed efficient esterification toward acetic acid and short- and medium-chain fatty acids (C₄-C₁₀). Among these fatty acids, esterese exhibited maximal activity (24 units) for hexanoic acid (C₆). However, its activity decreased rapidly (4 unit) for decanoic acid (C₁₀).

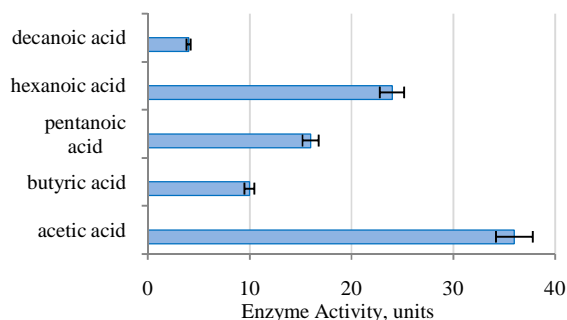


Fig. 1 Substrate specificity of esterase to carboxylic acids

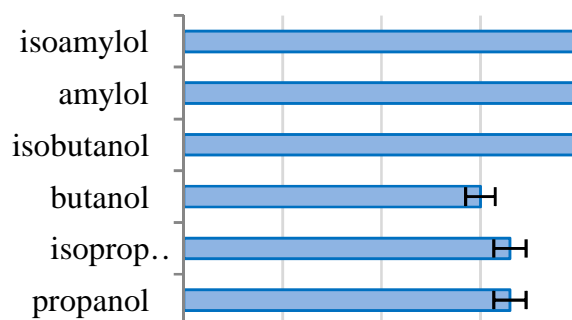


Fig. 3 Substrate specificity of esterase to alcohols

The esterase K_m value was determined by analyses of the slope and intercept for the plot (Lineweaver-Burk Diagramm) of the reciprocals of the steady-state versus fatty acids concentration (Fig 2). Thus, the initial concentration of the substrate was a limiting factor in the enzyme-substrate interactions. The minimal K_m value for the enzyme was found to be 0.6 mM for acetic acid. Consequently, the researched esterase is acetyl esterase. Regarding to fatty acids, the K_m value increased in the order C_6 (1.0 mM) < C_5 (1.2 mM) < C_4 (1.4 mM) < C_8 (1.8 mM) < C_{10} (2.5 mM). As shown in Fig 2, the esterase was more specific toward acetic acid and medium-chain alcohols, particularly to hexanoic acid, which is the most pungent odorant and toxic one among medium-chain volatile acids.

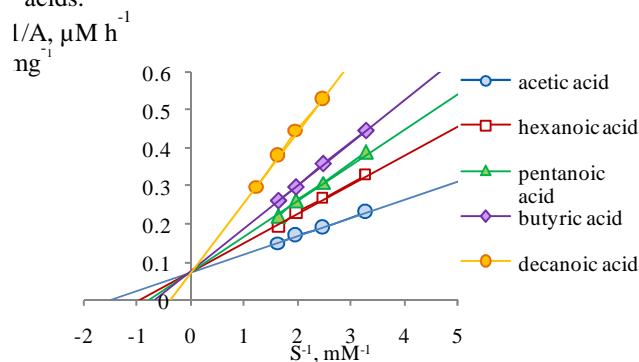


Fig. 2 Double-reciprocal plot of kinetic data on substrate specificity of esterase to carboxylic acids

The dependence of esterase activity on alcoholic substrate concentration in the reaction mixture measured from 0.2 to 0.5 mM (Fig 3). Investigations of the enzyme specificity to alcoholic substrates showed the best result with isoamyl alcohol (61 unit) in esterification reaction with acetic acid. Conversion with higher alcohols increased in the order C_3 (33 units) < C_4 (42 units) < C_5 (61 units). In addition, esterase reacted more specifically with branched alcohols such as isobutyl alcohol (42 unit) and isoamyl alcohol (61 units).

A high level of enzyme activity was observed when the initial concentration of acetic acid in the reaction mixture attained 0.5 mM. The Lineweaver-Burk plot (Fig 4) shows a minimal K_m value for the enzyme as 1.0 mM for isoamyl alcohol. Regarding to other alcohols K_m value decreased in the order isoamylol (1.0 mM) < amylol (1.2 mM) < isobuthanol (1.4 mM) < isopropanol (2.2 mM) < buthanol (4.0 mM) < ethanol (5.0 mM). Such effects were already noted in the past for other enzymes [10].

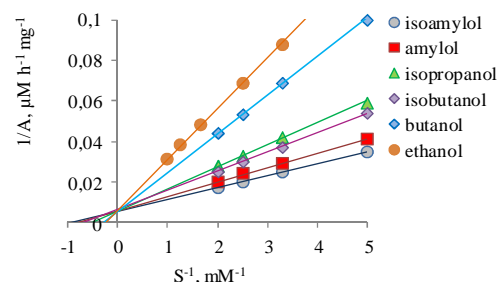


Fig. 4 Double-reciprocal plot of kinetic data on substrate specificity of esterase to alcohols

Thus, the application of esterase may have important practical implications, for the production of liqueur and other alcoholic beverages which contain 10-3000 mg/L volatile congeners (acetic acid, fusel alcohols) [11]. On the other hand, during the grape wine distillation large amount of higher (fusel) alcohols represented substantially by isobutyl and isoamyl alcohols as well as medium chain fatty acids such as hexanoic, octanoic and isobutanoic acids are found in distillates [12], [13]. While the concentrations are known to vary, it is well-known that these alcohols are highly toxic and pungent odourant. The presented results indicate that may be well source to be used for the treatment of alcoholic beverages such as vodka, liqueur, sake, brandy etc. The transformation of toxic fusel alcohols into volatile esters will support the production of the high quality products with reduced content of higher alcohols as well as with improved degustational and hygienic properties. As distinguished from traditional approach the presented acids and alcohols are esterified to form volatile esters which possess pleasant aroma. The esterase catalysed the esterification reaction very specific while other parameters remained unchanged.

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