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The Influence of Acyl Donators on The Enzymatic Activity of Lipase *Lipozyme CALB* in The Esterification Process

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ABSTRACT

An influence of the nature of aliphatic acid of C2 - C8 on the process of enzymatic esterification of butyl and benzyl alcohols was investigated. The process was carried out in hexane with molar ratio of acid: alcohol 1:2 for butanol and 1:1.1 for benzyl alcohol, at the temperature of reaction mixture of 35 °C. Lipase *Lipozyme CALB* in various concentrations was used. Process course was analyzed by conversion of the corresponding acid. It is established that the process of enzymatic esterification of aliphatic alcohol substrate (butanol) by the enzyme *Lipozyme CALB* is more efficient than that of benzyl alcohol, at use of identical acid substrates. It was found the most active donator of acyl group for esterification among studied aliphatic acid was acetic acid. The caprylic (n-octanoic) acid having bigger molecular weight and the weakest acidic properties was the weakest acyl donator, and the process of esterification with this acid runs the least efficiently. Results of experiments have shown that activity of aliphatic acids as acyl-donators in processes of an esterification dependent on nature of alcoholic substrate and is determined both by the steric factor, in this case length of a hydro carbonic chain of acid, and by the acid properties (value of pKa).

1. Introduction

Lower aliphatic acids and alcohols are widely used as food flavors, fragrances in perfumes and cosmetics, fragrances in the production of synthetic detergents.

Of the known chemical methods for the preparation of synthetic esters, the most widespread method is esterification, which occurs with the participation of acidic or alkaline catalysts at elevated temperatures. In recent years, the number of works on the enzymatic esterification of fragrant substances and the use of lipases has sharply increased, that is determined by a number of unconditional advantages of bio catalysis before chemical catalysis [1-7].

In the previous work we investigated an influence of the length and branching of the hydrocarbon radical of aliphatic alcohols C4-C11, as acyl group acceptors, on an enzymatic esterification using pancreatic lipase [8]. These results have shown that with increasing of length and branching of the hydro carbonic radical of alcohols enzymatic synthesis becomes more difficult.

Throughout these works an influence of another substrate of the reaction - acid - on the process of an enzymatic esterification has been studied. It have been used acetic (C2), propionic (C3), butyric (C4), valeric (C5), caproic (C6) and caprylic (C8) acids differing both in the length of the hydrocarbon substituent and in different acidity (PKa values).

2. Experimental Methods

An enzyme – *Lipozyme CALB*, production of Novozymes. Diapason of work - pH 5-9, temperature 30-60 °C, activity - 50-55 units/mg of protein (based on the hydrolysis of olive oil).

Used substrates – aliphatic acids (acetic, propionic, butyric, valeric, caproic and caprylic) and alcohols (n-butanol and benzyl alcohol) – were reagent grade. As the solvent it was used hexane (reagent grade).

The synthesis was carried out at 35 °C, the duration of the process varied from 0.5 to 7 hours. The amount of enzyme in the reaction mixture was 10 μL/mL, 20 μL/mL, 30 μL/mL. The acids were used in 0.1 N concentrations. Molar ratio acid: butanol 1:2, acid: benzyl alcohol 1:1.1.

Control of process of esterification was carried out by titration of residual acid with a 0.1 N alcoholic solution of NaOH (in 80% alcohol) in the presence of 0.05 mL of 1% alcohol solution of phenolphthalein as an indicator until a constant pink color obtaining. The acid conversion (B) was calculated by the formula: B = (K-O/K) 100%, where K is the amount of alkali solution that went to titration of the control, O is the amount of alkali solution that went into titration of the sample.

After the process was complete the enzyme was separated from the reaction mass, an excess of alcohol and hexane removed by vacuum distillation. The formation of the ether was confirmed by the IR spectrum recorded on a Fourier spectrometer "Infra-LUM F". All experiments were made in six replicates. The data were processed by means of applied software package statistica. The relative error was not more than 7%.

3. Results and Discussion

All experiments were carried out in hexane solution, since it is known that it has the lowest inhibitory effect on enzymes.

Curve dependences of conversion of acids on time at an esterification of benzylic alcohol with use of Lipozyme CALB enzyme are given in the Fig. 1. As can be seen from these data, the esterification process proceeds slowly, the maximum conversion of acetic acid after 4 hours of synthesis makes 52% and caprylic acid over the same period about 16%. Thus, at an esterification of benzyl alcohol containing in its structure an aromatic fragment the acylating ability of aliphatic acids at all stages of the process is determined by the length of hydrocarbon radical of the acid: the maximum conversion was in acetic acid case, and further decreasing of the activity till caprylic acid: C2> C3> C4> C5> C8.

Fig. 2 shows the kinetic curves of the butanol esterification process using the Lipozyme CALB enzyme at a concentration of 10 μL/mL. As can be seen from these data, the highest acylation rate of butanol throughout the process shows acetic acid with the maximum conversion after 6 hours of synthesis of 85%. The lowest acylation rate of butanol shows caprylic acid with the maximum conversion after 6 hours of synthesis of 61%. Conversion of caproic acid in 6 hours of the process is compared with the conversion of propionic acid and is 77%. The propionic, butyric and valeric acids acylate butanol almost equally at the first five hours of the process – around 73%, but then the conversion curves diverge.

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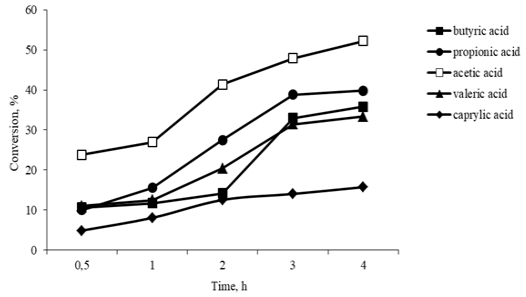

Fig. 1 The dependence of acid conversion on the time of the esterficatoin process of benzyl alcohol. Aliphatic acids was used as 0.1 N solutions, molar ration of acid:alcohol 1:1.1, the concentration of the enzyme *Liposyme CALB* 30 μL/mL, the temperature of reaction mixture 35 °C

With an increase of amount of the enzyme to 20 μL/mL, the process of acylation of butanol with different acids has a similar character as for the enzyme concentration of 10 μL/mL. However, the conversion is higher for all acids, from 3 to 6% (Fig. 3). Thus, acetic acid has a maximum conversion of 91%, butyric 78% (during 5 hours of synthesis), valerian - 89%, propionic and caproic - 80%, caprylic - 64% (during 6 hours of synthesis).

Increasing of the enzyme concentration to 30 μL/mL has led to some changes in kinetic characteristics (Fig. 4). Acetic acid still has the highest acylating ability, however, valeric acid is approaching it, and both acids give a maximum conversion of 93% within 6 hours. The minimum acylating capacity is shown by caprylic acid with a conversion maximum of 70% in 6 hours of the process. As to propionic, butyric, and caproic acids, they resulted in 83-87 % of conversion during 6 hours of synthesis.

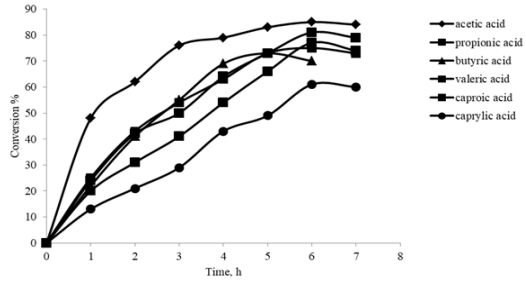

Fig. 2 The dependence of acid conversion on the time of the esterficatoin process of butanol. Aliphatic acids was used as 0.1 N solutions, molar ration of acid:alcohol 1:2, the concentration of the enzyme *Liposyme CALB* 10 μL/mL, the temperature of reaction mixture 35 °C

So, the effect of the nature of the acylating acid on the esterification of butyl alcohol shows a more complex character of the dependence. Probably, the process of acylation of an aliphatic alcohol, which proceeds more efficiently than the acylation of benzyl alcohol, is influenced not only by the spatial factor, but also by the acid properties of the donators, their ability to deprotonation. If to compare the used acids in their PKa, that are close on the value, they not absolutely correspond to their molecular masses, i.e. length of hydro carbonic chains [9]. Acids C5,C6,C3 are practically equal in their proton ability, despite the varying length of the hydrocarbon substituents: Acid C2 > C4 > C5 ≈ C6 ≈ C3 > C8

PKa 4.75 4.82 4.86 4.86 4.87 4.89…

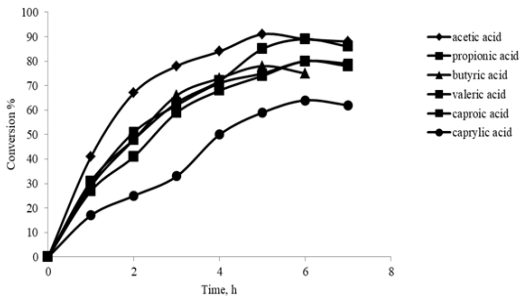

Fig. 3 The dependence of acid conversion on the time of the esterficatoin process of butanol. Aliphatic acids was used as 0.1 N solutions, molar ration of acid:alcohol 1:2, the concentration of the enzyme *Liposyme CALB* 20 μL/mL, the temperature of reaction mixture 35 °C

Probably, both of these factors, the values of PKa and the spatial effect, i.e. the length of the hydro carbonic chain of the acid, affects their acylating ability. The nature of the alcohol substrates influences the esterification process also. The presence of an aromatic fragment (benzyl alcohol) in the substrate structure leads to a slowing down of the esterification process, as was previously shown for the processes of enzymatic hydrolysis [10]. At low rates of esterification, when alcohol contains an aromatic fragment, the spatial effect of the acyl donators is decisive. However, with more effective esterification for an aliphatic alcohol substrate both the factor PKa and the length of the hydrocarbon radical affect the process.

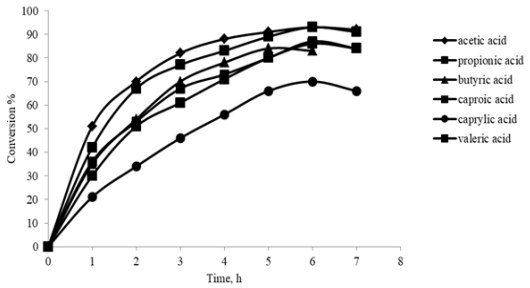

Fig. 4 The dependence of acid conversion on the time of the esterficatoin process of butanol. Aliphatic acids was used as 0.1 N solutions, molar ration of acid:alcohol 1:2, the concentration of the enzyme *Liposyme CALB* 30 μL/mL, the temperature of reaction mixture 35 °C

The formation of ethers was confirmed by the IRS method. So, on spectra of benzyl propionate a broad intensive stretching vibrations of ester group -C-O-C- appeared at 1171.6 cm^{-1} region, the stretching vibrations of the carbonyl group $\upsilon_{C=0}$ 1736.5 cm^{-1} was shifted on 28 cm^{-1} in comparison with $\upsilon_{C=0}$ 1708.5 $cm^{-1}$of the initial propionic acid. The butyl valerate spectrum also has a stretching vibrations of ester group at 1173.9 cm^{-1} and stretching vibrations of the carbonyl group $\upsilon_{C=0}$ at 1735 cm^{-1} which is shifted on 34 cm^{-1} in comparison with $\upsilon_{C=0}$ of the original valeric acid, 1701 cm^{-1}. These facts, as well as the absence of stretching vibrations of hydroxyl groups on the spectra of esters, confirm the formation of esters and their purity.

4. Conclusion

It is found that the esterification of butyl alcohol proceeds more effectively, than benzyl alcohol under the studied conditions. At esterification of benzyl alcohol by aliphatic acids of C2 – C8 series using the enzyme *Lipozyme CALB* an acylating ability of the acids in the studied conditions decreased from acetic acid to caprylic acid in accordance with the length of the hydrocarbon chain of acyl. At an esterification of butanol with the same series of aliphatic acids, the acylating ability has more complex character and is defined not only by the length of hydro carbonic chain, but also by the value of the PKa of the acids. Thus, the acylating ability of aliphatic acids in esterification processes depends on the nature of the alcohol substratum and is determined both by the length of the hydrocarbon chain and by their acid properties.

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