Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 7, No. 3, pp. 259-262, 1997

0960-894X/97 $17.00 + 0.00

PII: S0960-894X(96)00614-2

LIPASE-CATALYSED ESTERIFICATION IN SUPERCRITICAL CARBON DIOXIDE AND IN HEXANE.

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Abstract: Isoamyl acetate was synthesised in high yields (>90%) via lipase catalysed acylation of the corresponding alcohol by ammonium acetate in supercritical carbon dioxide (SCCD) and by acetic acid in hexane. The esterification rate was higher in hexane. The product yield depended sharply on the reagent concentrations in hexane whereas it was nearly independent on the pressure and temperature of the SCCD.

There is a great interest in the technological use of the bioconversion of chemical compounds in low-water solvents [1-4]. Elimination of water from the reaction medium enables the 'reversal' of the hydrolytic enzymes and their use for catalysis of synthetic reactions. A large number of commercially interesting compounds (pharmaceuticals, food additives, etc.) and their precursors are poorly soluble in water and their effective transformation can be carried out only in the presence of other solvents. Among potentially interesting near-anhydrous solvents, supercritical carbon dioxide (SCCD) seems to be very suitable for the enzymatic synthesis [2,5]. Supercritical fluids have a number of advantages with respect to organic solvents, including high diffusion and mass transfer rates essential for immobilized enzymes, easy product recovery without a trace of solvent, high solvent power and the possibility to change the properties of the solvent continuously by pressure and temperature changes [2,5,6]. Attractive features of SCCD also include nontoxicity, nonflammability, availability in large amounts at low cost, low environmental impact and a low critical temperature [2,5]. Several enzymes including lipases exhibit stable activity in SCCD [2].

Aroma compound synthesis is considered as one of future application of enzymatic reactions in SCCD [4]. The enzymatic ester synthesis offers several important advantages especially when esterification of a labile alcohol is concerned or an optically pure isomer is required as a reaction product[1,2,4]. However, biotechnological synthesis of simple flavouring esters has also potential interest for food industry, since the product may obtain natural label [1,7].

In this paper the lipase-catalysed synthesis of isoamyl acetate in SCCD and organic solvents has been studied [8]. Isoamyl acetate is widely used in food industries because of its characteristic banana flavour. On the other hand, acetates are suitable model compounds for the study of lipase-catalysed esterification, since acetates are not so easily synthesised by lipases in organic solvents as their superior homologues propionates, butyrates etc., for which the yields attain around 100% [9]. It should be noted here that the lipase-catalysed oxygen exchange in fatty acids and triglyceride hydrolysis proceed with comparable rates [10], suggesting that the enzyme

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acylation by free acids should be as fast as the acylation by ester substrates. This unique property, together with appropriate substrate specificity, makes lipases more suitable catalysts for esterification than other hydrolytic enzymes.

Table. Lipase-catalysed synthesis of isoamyl acetate in SCCD.

[i-AmOH], M	[I-AmOH] $[NH_4Ac]$	Rate, mmole g^{-1} h^{-1}	Yield, %	Pressure, bar	Temp, °C	Enzyme
0.20	1.1	0.33	88	200	40	Novozym
0.20	1.1	0.30	90	200	60	"
0.90	1.1	0.21	74	200	40	"
0.11	1.1	0.02	71	150	40	Lipolase
0.11	1.1	0.025	80	200	40	"
0.20	1.1	0.06	80	200	40	"
0.20	2.0	0.06	96	200	40	"

The data on the lipase-catalysed esterification of isoamyl alcohol in SCCD are summarised in Table. Both lipases catalysed the esterification only when ammonium acetate was used as acyl-donor, no ester was formed when the acetic acid was added to the reaction mixture. It has been shown that various lipases catalyse the esterification of alcohols by long-chain free acids [11]. Lipolase also catalysed the acylation of ethanol with water insoluble free C8 acid: 90% of C8 acid (0.01 M) was converted to the corresponding ethyl ester in SCCD at 130 bar and 40°C in 5 hours in the presence of 0.5 g of the enzyme. This suggests that the salts of highly water soluble acids should be used as the acyl-donors instead of free acids when the reaction is performed in SCCD. It seems probable that the presence of the ammonium ions is necessary in order to maintain the pH in the microaqueous phase surrounding the enzyme not upon dissolving carbon dioxide but upon dissolving the highly soluble acid.

The acetate was almost completely converted to the corresponding ester at the alcohol/acid ratio 2. Novozym was more efficient catalyst of esterification than Lipolase. The product yield and conversion rate were only slightly smaller at higher reagent concentrations and lower pressure, whereas they did not depend on the reaction temperature in the range from 40 to 70°C. It has been shown that supercritical carbon dioxide covalently modifies the amino groups of the proteins at lower temperatures whereas at temperatures higher than 55°C these complexes dissociate [12]. This process did not affect the enzyme activity in our case.

The highest yield for the enzymatic synthesis of isoamyl acetate 80% reported in literature [9] was achieved at alcohol/acid ratio 4 in n-heptane using *Mucor miehei* lipase as a catalyst. In SCCD the presence of isoamyl alcohol in only a 10% excess was necessary to achieve this ester yield.

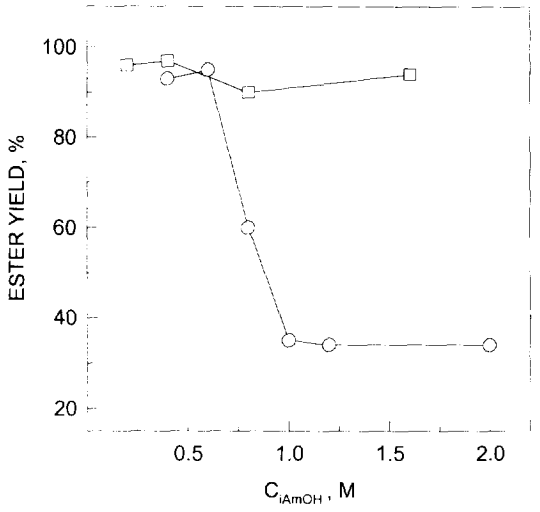

Figure 1. Influence of reagent concentrations on the isoamyl acetate synthesis catalysed by Novozym in hexane at alcohol to acid ratios 1.1 (O) and 0.9 (□). Product yields correspond to the conversion of isoamyl alcohol (□) and acetic acid (O).

We also studied the lipase-catalysed esterification in hexane, which is reported to be the most suitable organic solvent for lipase-catalysed esterification [3]. At low reagent concentrations the product was observed in a high yield (95%) in the equimolar mixture of reagents. However, as shown in Figure 1., the product yield was substantially decreased at high reagent concentrations. It has been assumed that acetic acid may inhibit the enzyme activity in the organic solvents [9]. A continuous decrease in the initial rate of esterification with increasing reagent concentrations from 16 mmole g^{-1} h^{-1} in 0.2 M solutions to 7 mmole g^{-1} h^{-1} at 2 M tended to confirm this suggestion. However, the high ester yield was restored in a small excess of isoamyl alcohol suggesting that the decrease in the ester yield was not caused by straightforward enzyme inhibition by acetic acid. It seems probable that the ester yield is determined by the content of microaqueous phase in the vicinity of the enzyme. The ester yield exceeded 90% also in a continuous flow process under similar conditions where the 2M solution of reagents in hexane was passed through a column containing 0.25 g of Novozym with flow rate 0.25 ml/min.

The product yield in SCCD exceeded the highest value reported in the literature for enzymatic isoamyl acetate synthesis. However, the results tend to indicate that in spite of its convenient characteristics SCCD may not be the most efficient solvent for biocatalysis.

Acknowledgements. This work was supported by the Estonian Science Foundation (Grant No. 549). We gratefully acknowledge Novo Nordisk for the gift of the enzymes.

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8. Reaction was carried out in a stainless steel reactor with internal volume 100 ml.

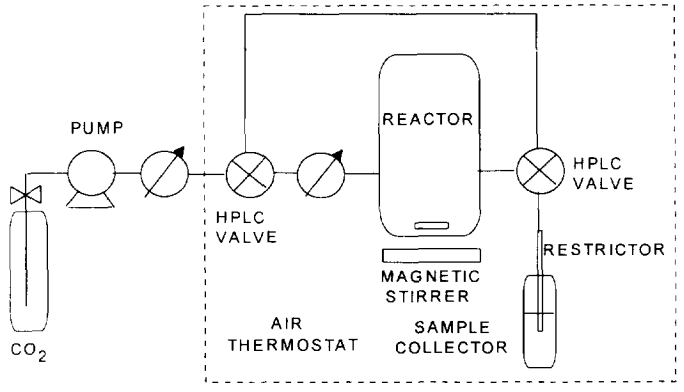

Figure 2. Apparatus for enzyme-catalysed esterification in SCCD.

Before each experiment the reactor was opened and a precise amount of the substrates, water and the enzyme was introduced within the reactor. After sealing, the system was pressurized by pumping liquid carbon dioxide (99.5 % pure Eesti AGA, Estonia).by a syringe pump (DuPont Instruments, USA) to the desired pressure and the reactor was isolated from the circuit by closing valve 1. Samples were withdrawn from the reaction mixture and depressurized through a frit restrictor (AS Englo, Estonia). Samples were collected into methanol. Two HPLC valves in the system enabled to wash the restrictor between sample collecting.

Esterification in *n*-hexane was carried out in a screw capped flask containing 10 ml of the solvent. The mixture was stirred on a magnetic stirrer at room temperature.

Samples of the reaction media were periodically analysed by a gas chromatograph Chrom 5 (Laboratorni Pristroje, Praha, Czechia) equipped with a capillary column Nordion NB-20M (0.5μ, 25 m x 0.32 mm) and a FID detector at 150°C. Helium (0.5 ml/min) was used as a carrier. The temperature gradient was from 55°C to 120°C at 10°C/min. The molar ratio of isoamyl acetate and isoamyl alcohol was determined.

The lipolytic enzymes, Novozym and Lipolase were kindly provided by Novo Nordisk, Denmark.

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(*Received in Belgium* 29 *October* 1996; *accepted* 16 *December* 1996)