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Journal of Molecular Catalysis B: Enzymatic 11 (2001) 949–954

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

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Influence of water activity and water content on sugar esters lipase-catalyzed synthesis in organic media

F. Chamouleau [a], D. Coulon [a,b], M. Girardin [a], M. Ghoul [a,*]

[a] *Lab. des Sciences du Genie Chimie, Groupe des Lipoprocédés ENSAIA, 2 avenue de la forêt de Haye, Vandoeuvre les Nancy 54500, France*

[b] *ESTBB, Université de Bordeaux 2, 146 rue Léo Saignat, Bordeaux 33076, France*

Abstract

Lipase-catalysed ester bond synthesis in organic media was directed by thermodynamic water activity of the reaction mixture. The effects of water activity and water content were investigated with fructose palmitate synthesis catalysed by *Candida antarctica* lipase. Conversion yield, initial rate of synthesis and reaction selectivity were analysed. Initial water activity of the reaction medium was fixed at a desired value by pre-equilibration with saturated salt solutions. *C. antarctica* lipase activity depended strongly on initial water activity value. Conversion yield and initial rate decreased with the increase of water activity. Whatever the a_w value, fructose monopalmitate was only synthesised. The best results were achieved for a reaction medium with an initial water activity less than 0.07. In these conditions, 28.5% of fructose was acylated in fructose monopalmitate with an initial rate of 4.9 g $l^{-1}h^{-1}$. To improve the process, the effect of the water was determined by drying the medium with molecular sieves. Conversion yield of fructose and initial rate of fructose monopalmitate synthesis were raised, respectively, to 73.4% (32 g l^{-1}) and 10.1 g l^{-1} h^{-1}. However, the use of dried medium affected the selectivity of the reaction. In these conditions, both mono- and di-fructose palmitate were synthesised (16.7 g l^{-1}). © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Water activity; Esterification; Water content; Sugar esters; Lipase; Selectivity

1. Introduction

Sugar esters are non-ionic surfactants which can be synthesised either by chemical or enzymatic processes [1–4]. Chemical methods have been developed since 1950 [5], whereas enzymatic synthesis has begun only in 1986 [6]. Due to the high regiospecificity of enzymes, enzymatic synthesis is characterised by the production of a more defined product (sugar mono ester), whereas chemical process usually leads to a mixture of sugar polyesters. Sugar esters can be used as surface-active components in many industrial scopes as cosmetics, pharmaceuticals and food industries [7].

Enzymatic synthesis in organic medium is based on the ability of proteases and lipases to catalyse reverse hydrolysis, i.e. the formation of peptidic and ester bonds, respectively. These reactions take place

* Corresponding author. Tel.: +33-3-8359-5892; fax: +33-3-8359-5796.

E-mail address: ghoul@ensaia.u-nancy.fr (M. Ghoul).

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PII: S1381-1177(00)00166-1

in a medium presenting a low water activity [8,9]. In these conditions, the thermodynamic equilibrium of the reaction is shifted towards synthesis reaction instead of hydrolysis [10–12]. However, direct esterification of a carboxylic acid with a hydroxyl group leads to the production of water. This by-product is thermodynamically unfavourable to ester synthesis. It will be removed from the medium to assure a high synthetic activity.

In the literature, the effect of water on enzymatic synthesis carried out in organic medium has been studied in regard with water content or water activity [13–15]. However, there are only a few works that investigated these parameters in the case of sugar ester synthesis.

In this study, both effects of water activity and water content on sugar ester synthesis were investigated. Direct esterification reactions were catalysed by an immobilised *Candida antarctica* lipase. Fructose and palmitic acid were used as substrates. The conversion yield, initial rate and selectivity of the reaction are used as response factors to characterise the behaviour of the reaction.

2. Materials and methods

2.1. Materials

Syntheses of fructose palmitate were performed in a double jacket batch reactor (250 ml) using immobilised *C. antarctica* lipase as biocatalyst (Novozym, Novo Industri). 2-Methyl 2-butanol (Merck, ref. 806193) was used as solvent, fructose (Merck, ref. 4007) as acyl acceptor and palmitic acid (Fluka, ref. 76122) as acyl donor.

2.2. Water activity pre-equilibration of reaction medium

Before the start of the reaction, the enzyme and the medium-containing fructose, palmitic acid and 2-methyl 2-butanol were pre-equilibrated separately with the water vapour of saturated salt solutions. Pre-equilibration was done at 25°C for 5 days. The saturated salt solutions used were prepared with LiCl (water activity, $a_W = 0.11$), CH_3COOK ($a_W = 0.23$), $MgCl_2$ ($a_W = 0.33$), $Mg(NO_3)_2$ ($a_W = 0.53$) and NaCl ($a_W = 0.75$).

2.3. Water activity determination

Water activities of the reaction medium and biocatalyst were determined using a Thermoconstanter TH200 Novasina (20°C) and the water content of the liquid phase was measured with a Metrohm 737 KF coulometric Karl Fisher apparatus.

2.4. Determination of isotherm adsorption curve

The water adsorption curve was determined with the oven 707 KF Metrohm coupled with the coulometric Karl Fisher apparatus (Metrohm 737 KF). The optimal temperature and extraction time were 140°C and 2500 s, respectively. The quantity of water extracted was recorded every 5 s until the destruction of catalyst. For each sample, the water extraction was done three times.

2.5. Sugar ester synthesis operating conditions

Syntheses were carried out in 200 ml of 2-methyl 2-butanol. An equimolar mixture of fructose and palmitic acid (0.139 M) was prepared. Direct esterification was catalysed by 5 g l^{-1} of Novozym. The temperature was fixed at 60°C and stirring rate at 100 rpm. The agitation of the reaction medium was assured by a suspended magnetic stirrer to avoid enzyme support destruction. The reactions were started by mixing both pre-equilibrated phases (reaction medium and immobilised enzyme). For the control, the same experimental conditions were applied without a pre-equilibration period.

2.6. Synthesis with dried medium

In these experiments, all components of the reaction medium were dried before use. 2-Methyl 2-butanol was dried with 100 g l^{-1} molecular sieves 4A for 5 days. Substrates and enzyme were dried on silica gel, in a dessicator, under vacuum for 1 week. After drying, all components were mixed in the

reactor, with 100 g l^{-1} molecular sieves previously heated for 2 h at 250°C. Stirring rate was increased at 250 rpm.

2.7. Analysis

The time course of fructose palmitate synthesis was monitored by quantitative TLC/photodensitometer and high-performance liquid chromatography (HPLC). About the TLC/photodensitomer method, samples (5 μl) were set on Kieselgel G60 plates (Merck, ref. 5553). The plates were eluted in a mixture of $CHCL_3$/MeOH/AcOH/H_20 (80:15:8:2 v/v/v/v). Then plates were sprayed with a α-naphtol/sulphuric reagent before heating at 105°C for 5.5 min. Sugars and sugar esters appeared as purple spots and their concentrations were determined at 545 nm using a photodensitometer CS 9000 (Shimadzu).

HPLC analyses were realised on Lichrospher 100 RP 18 column 5 μm with a scattering mass light detector (Eurosep, DDL 31). The different components were separated using a gradient of methanol and water.

3. Results and discussion

3.1. Enzymatic synthesis of fructose palmitate

As a standard, the enzymatic synthesis of fructose palmitate is realised with an equimolar mixture of fructose and palmitic acid. Under these conditions, the initial water activity of the medium is around 0.07. Fructose is used in excess: the solubility of fructose in 2-methyl-2-butanol at 60°C is about 11 g l^{-1} while palmitic acid is completely soluble. During the reaction, the excess of fructose is progressively solubilised in parallel with fructose acylation [16]. In these conditions, the reaction leads only to fructose monopalmitate. In the medium, the fructose can be available as furanose with two primary alcohols (C1 and C6 position) or pyranose with one primary alcohol (C1 position). So, the fatty acid can be linked to the two anomer forms (α and β) of the sugar on the different positions of primary alcohols.

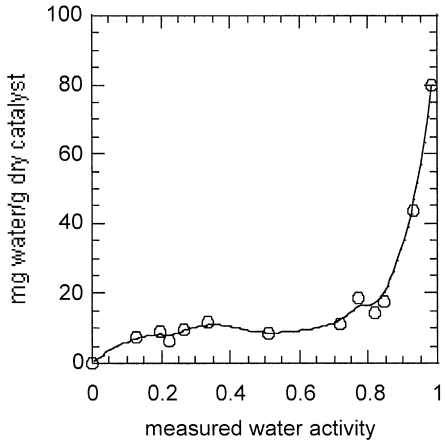

Fig. 1. Water adsorption curve for Novozym 435 at 20°C.

In a previous paper [17], we have found that oleyl-palmitate fructose synthesised by *C. antarctica* is formed by four isomers (α and β palmitoyl-6-fructofuranose, palmitoyl-1-β-fructofuranose and palmitoyl-1-β-fructopyranose). Arcos et al. [18] have reported similar results when acetone is used as organic solvent. At the equilibrium, the conversion yield is about of 28.5% (16.5 g l^{-1} of fructose monopalmitate) with an initial reaction rate of 4.9 g l^{-1} h^{-1}. This low value of the conversion yield can be explained by the water production during synthesis. In fact, the water adsorption curve (Fig. 1) shows that a small amount of additional water can raise drastically the water activity of the immobilised enzyme. So the thermodynamic equilibrium favoured the hydrolysis reaction instead of direct esterification.

3.2. Influence of the initial water activity

Reaction medium and immobilized *C. antarctica* lipase are pre-equilibrated separately at a desired a_W using saturated salt solutions (from 0.11 to 0.75). After 5 days, enzyme and reaction medium are mixed and the synthesis of fructose palmitate is followed. The resulting reaction rate and steady state concentration in function of a_W are reported in Fig. 2. They decrease when the initial water activity raises. The decrease of fructose palmitate steady state concentration can be explained by the role of water during synthesis as substrate. In fact, water replaces the

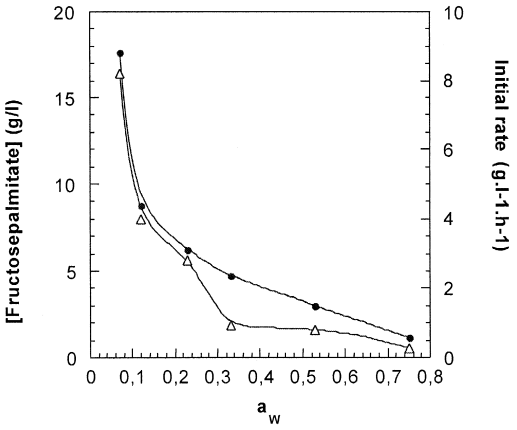

Fig. 2. Influence of initial water activity of fructose monopalmitate steady state concentration (●) and initial rate (Δ).

hydroxyl group of fructose as acyl acceptor. So for high a_W value, the equilibrium of the reaction is shifted towards hydrolysis, leading to low ester concentration. This competition between water and fructose affects also the initial rate of synthesis

The decrease of initial rate for high water activity can be also explained by a limitation of palmitic acid transport from the reaction medium to the vicinity of enzyme due to water. In fact, electronic microscopy observations of immobilised enzyme at different a_W values have shown that water surrounded the particle of the biocatalyst [19], forming a layer preventing lipophilic substrate access to the enzyme, and led to particle aggregation. Even if we do not use the same resin, a similar effect can be supposed.

According to our results, it appears that the lower the water activity is, the higher is the synthetic activity of *C. antarctica* lipase. Best conversion yield was achieved for a_W less than 0.07. For a_w value lower than those of a saturated LiCl solution ($a_W = 0.11$), it is not possible to reduce more the water activity of the medium using the pre-equilibration technique developed by Goderis et al. [21], unless adding directly in the medium salt hydrate pairs. To improve the enzymatic acylation process, the effect of the water content was investigated by drying the reaction medium with molecular sieves.

3.3. Synthesis with molecular sieves

The different components involved in the reaction medium were dried several days. The obtained a_W values are reported in Table 1.

The enzymatic support is the most affected by the drying process. The water activity decreases from 0.43 to 0.13. The absence of variation of the a_W of the medium before and after drying is due to the fact that the ratio of the solvent is more than 90% of the medium.

The following syntheses have been performed with dried components and with molecular sieves 4A in the reactor to remove the produced water. In these conditions, the steady state concentration of fructose palmitate is raised from 16.5 g l^{-1} to more than 32 g l^{-1}. The conversion yield of fructose in fructose monopalmitate is 55.1%. In addition, the initial rate of fructose monopalmitate synthesis is twice higher compared to control (from 4.9 to 10.1 g l^{-1} h^{-1}) (Fig. 3).

This result is due mainly to a shift of the reaction towards synthesis. Water is kept by the molecular sieves, and a larger amount of sugar esters is needed to reach the equilibrium.

These results are in accord with the data reported by Mutua and Akoh [20]. These authors have studied the effect of water addition on enzymatic acylation of methyl-glucoside by oleic acid methyl ester. Water addition provoked a decrease of conversion yield

Table 1
The a_W values of the different components of reaction medium determined at 25°C with the Thermoconstanter TH 200 (Novasina)

Component	Solvent	Fructose	Palmitic acid	Enzyme	Medium
a_W before drying	0.07	0.41	0.32	0.43	0.07
a_W after drying	0.07	0.36	0.31	0.13	0.07

from 76.6% to 13.3% when 1.5% water was added. This profile was also mentioned with *Rhizomucor miehei* lipase [22]. However, the influence of water depends on the origin of lipase. In fact, Janssen et al. [23] have realised sorbitol esterification in a mixture of 2-pyrrolidone and phosphate buffer. For water concentration lower than 0.2 M, *Chromobacterium viscosum* lipase did not catalyse the reaction. Highest activity was achieved for water concentration between 0.4 and 1 M. For higher values, conversion yield declined [23]. For *Chromobacterium* lipase immobilised on celite and *Pseudomonas* lipase immobilised on XAD-4, synthetic activity was improved for water content lower than 1.5% and 0.5%, respectively [24].

The use of a drying agent affects also the selectivity of *C. antarctica* lipase (Fig. 4). Whereas only fructose monopalmitate is produced without molecular sieves, the addition of molecular sieves leads to the synthesis of fructose dipalmitate (about 16.7 g l^{-1}). This concentration corresponds to 18.3% of the initial sugar pool acylated in fructose dipalmitate. The global substrate conversion yields are 73.4% and 91.7% for fructose and palmitic acid, respectively.

The loss of selectivity may be explained by the reduction of the hydration layer surrounding the proteins. In fact, the lack of water near the enzyme provokes an increase of the hydrophobicity and consequently, the decrease of fructose solubility. In these conditions, monopalmitate becomes a better substrate than the fructose.

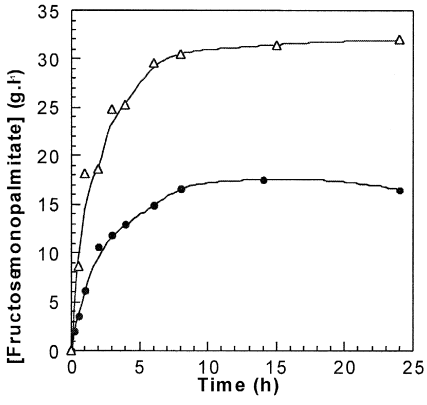

Fig. 3. Influence of drying of reaction medium on fructose monopalmitate synthesis. Reaction performed without (●) or with (Δ) molecular sieves.

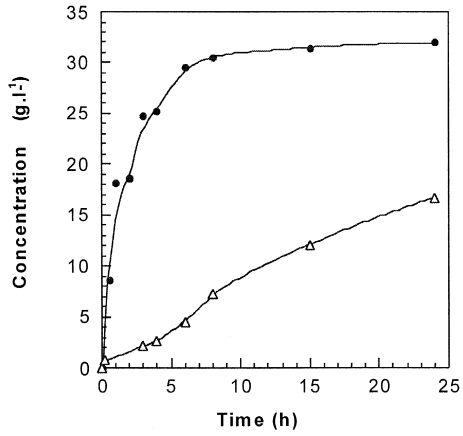

Fig. 4. Kinetics of fructose monopalmitate (●) and fructose dipalmitate (Δ) for a synthesis realised with addition of molecular sieves.

4. Conclusion

The initial water activity strongly affects fructose direct esterification by palmitic acid catalysed by immobilised *C. antarctica* lipase. The lowest water activity (0.07) led to the highest conversion yield (28.5%) and initial rate (4.9 g l^{-1} h^{-1}). The use of molecular sieves increases the performances of the reaction. However, the selectivity of the enzyme is affected. The increase of conversion yield and initial rate due to the reduction of water activity can be explained by the adsorption curve of Novozym 435, which indicates that a small increase of water amount provokes a wide variation of a_w. The loss of the enzyme selectivity in the presence of molecular sieves is due to the fact that this agent removes water in the microenvironment of the enzyme and consequently, the hydrophobicity increases. The solubility of fructose is lowered and monopalmitate is preferentially used as substrate. In the presence of the drying agent, thermodynamic equilibrium of the reaction is also shifted towards ester hydrolysis instead of ester synthesis.

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