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Enzymatic acylation of a bifunctional molecule in 2-methyl-2-butanol: Kinetic modelling

Eric Husson[a], Veronica Garcia-Matilla[a], Catherine Humeau[b], Isabelle Chevalot[a], Frantz Fournier[a], Ivan Marc[a,*]

[a] LRGP - UPR 3349 CNRS, Nancy Université, 2 avenue de la forêt de Haye, 54500 Vandœuvre-lès-Nancy, France
[b] LIBio, Nancy Université, 2 avenue de la forêt de Haye, 54500 Vandœuvre-lès-Nancy, France

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ABSTRACT

The kinetics of enzymatic acylation of a bifunctional molecule exhibiting both an amino and a hydroxyl function, considered as a peptide-like model molecule, is studied. The acylation of 6-amino-1-hexanol by ethyl oleate in 2-methyl-2-butanol catalysed by Novozym 435® has been investigated and modelled. This acylation process generated two products issued respectively from the O-acylation of the 6-amino-1-hexanol, and from the N-acylation of the O-acyl product, which leads to the N,O-diacyl product. Experimental results confirmed the sequential reaction scheme. An experimental approach was adopted to identify the impact of several operating conditions on the reaction performances, such as initial substrates molar ratio and stirring rate. The kinetics of enzyme deactivation was also studied and included in the kinetic model. The establishment of the kinetic model was based on three major hypotheses: (i) a sequential bi bi ping-pong enzymatic mechanism, (ii) an interaction between acyl-acceptor and the acyl-enzyme formation steps, (iii) the absence of influence of the residual hydrolysis of ethyl oleate on the kinetics. Under this hypothesis, the kinetic parameters of the model were quantified using a genetic-evolutionary algorithm as an innovative identification method. The simplified model made it possible to describe the kinetics of the 6-amino-1-hexanol acylation for some ranges of 6-amino-1-hexanol/ethyl oleate molar ratios (2/1, 1/1, 1/2, 1/4) with a single kinetic parameter set. The case of a significant excess of 6-amino-1-hexanol (4/1) or ethyl oleate (1/8) constituted the limit of the kinetic model. However, these extreme molar conditions are not suitable to develop bioconversion processes involving bioactive peptides that are not available in large quantities.

1. Introduction

The acylation process of peptides allows to enhance the lipophilic properties of these molecules, and to improve both their transport across biological membranes and their stability, as described in several works concerning the acylation of insulin and carnosine [1–5]. In a few cases, the covalent attachment with fatty acid may maintain or improve the therapeutic or biological activities of peptides [6,7]. The use of chemical processes to synthesize acylated peptides requires drastic reaction conditions and numerous functions protection and/or activation steps. Lipase-catalysed acylation in organic solvent constitutes a promising alternative approach [8–12]. As peptides are generally polyfunctional molecules, their selective acylation constitutes a challenge which depends on both the chemo-selectivity and the regioselectivity of the reaction. Thus, understanding the enzyme efficiency and selectivity toward N- or O-acylation is necessary before extending such conventional enzymatic processes to peptides acylation. Several studies investigated the enzymatic acylation of amino alcohols such as ethanolamine, diethanolamine or 6-amino-1-hexanol [13–17]. Concerning the lipase-catalysed acylation of 6-amino-1-hexanol in organic solvent, the reaction led to the synthesis of two products: the O-acyl product and the N,O-diacyl product. To explain these observations, the authors suggested that the reaction could be sequential. The two products might result from a first O-acylation of the 6-amino-1-hexanol and then from the N-acylation of the O-acyl product, leading to the N,O-diacylated product [17]. Until now, to our knowledge, modelling of the enzymatic acylation of a bifunctional molecule has not yet been studied. The establishment of a kinetic model would help to verify such hypothesis and to qualify the most suitable operating conditions. In the literature, only models concerning the lipase-catalysed acylation of primary alcohols in organic solvents have already been developed. Most are based on the well-known bi bi ping-pong mechanism, characterised by the formation of an acyl-enzyme intermediate, and were used to describe O-acylation reactions catalysed by the lipase B of *Candida*

* Corresponding author.
E-mail address: Ivan.Marc@ensic.inpl-nancy.fr (I. Marc).

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antarctica (CAL B) in an organic solvent [18,19]. The kinetic modelling of O-acylation processes catalysed by other enzymes like the lipases of *Mucor miehei* and *Pseudomonas cepacia* was also based on this mechanism [20–22]. The bi bi-ordered mechanism, characterised by the formation of a tertiary complex, has been used in very few studies concerning trans-esterification reactions in solvent-free systems catalysed by the CAL B [23,24]. As the stability of the enzyme is in most cases a key issue in enzymatic processes, enzyme deactivation was taken into account, allowing to improve the agreement between simulated and experimental data [25–27].

The aim of the present work is to investigate the trans-acylation reaction of a bifunctional model molecule, the 6-amino-1-hexanol, with ethyl oleate in 2-methyl-2-butanol at 55 °C catalysed by the immobilized lipase B of *C. antarctica*. 6-amino-1-hexanol exhibits two potential acylable sites: one primary amino group and one hydroxyl group. The presence of these two functional groups led us to consider this substrate as a peptide-like model. In the first step, an experimental approach is performed to study the influence of substrates molar ratio and stirring rate conditions on the performances of the reaction. The deactivation of the lipase during the incubation time in 2-methyl-2-butanol at 55 °C was also investigated. In the second step, a kinetic model based on this experimental approach was developed and the kinetic parameters were identified using a genetic-evolutionary algorithm [28,29]. The most suitable model was proposed and validated for several substrates molar ratios by comparing model predictions to experimental results. In the final section, the limits of this model are discussed.

2. Materials and methods

2.1. Chemicals and enzyme

6-Amino-1-hexanol (97%) and ethyl oleate (99%) were purchased from Sigma–Aldrich (Steinheim, Germany). Novozym 435® (a lipase B from *C. antarctica* immobilized on an acrylic resin, E.C. 3.1.1.3) with propyl laurate synthesis activity of 7000 PLU g^{-1} and protein grade of [1–10%] came from Novo Nordisk A/S (Bagsraerd, Denmark). In this study 7000 PLU g^{-1} corresponds to 1 UA (arbitrary unit). 2-Methyl-2-butanol, methanol, trifluoroacetic acid (TFA) with 99% of purity were acquired from Carlo Erba (Rodano, Spain).

2.2. Acylation procedure

The enzymatic trans-acylation reactions of 6-amino-1-hexanol in organic solvent were carried out in a batch stirred reactor (Wheaton®) of 50 mL volume equipped with a condensation system to prevent solvent evaporation. In a typical acylation reaction, 6-amino-1-hexanol and ethyl oleate was added to 10 mL of 2-methyl-2-butanol previously dehydrated on 4 Å molecular sieves. The initial water activity of the media was quantified with a thermoconstanter Novasina® (Switzerland) and was inferior to 0.1. Reactions were performed at different stirring rates at 55 °C during 96 h. After the total dissolution of the substrates over 12 h, the acylation of 6-amino-1-hexanol was started by the addition of 10 g L^{-1} of lipase preparation. During the enzymatic reaction, samples of 50 μL were withdrawn, filtered then diluted 20× with methanol/TFA (100/0.1, v/v). The substrates and the products of the reaction were separated and quantified by HPLC (injection volume of 2 μL). To validate the repeatability of the experiments, each reaction was performed in triplicate and results were expressed as mean values with standard deviations. The conditions used for the purification and the identification of the acylated products by mass spectrometry and nuclear magnetic resonance, were described in a previous study [17].

To confirm that no chemical reaction occurred between 6-amino-1-hexanol and ethyl oleate, control experiments without enzyme were also carried out and showed the absence of product whatever the applied experimental conditions.

2.3. Analyses by HPLC

The time course of each reaction was monitored using HPLC (LC 10 AD – VP, Shimadzu, France) equipped with a UV detector at 214 nm and a light-scattering low temperature evaporative detector (Shimadzu, France) in this order. The column was a newly developed C18 amide 125 × 2.1 mm – 5 μm (Altima®, Altech, France) maintained at 25 °C. The mobile phase (0.2 mL min^{-1} flow rate) consisted in methanol/water/TFA (80/20/0.1, v/v/v). A constant elution gradient was applied to reach methanol/TFA (100/0.1, v/v) after 5 min. This methanol concentration was maintained for 18 min then gradually lowered to reach the initial methanol/water ratio at the end of the run (i.e. 34 min). Calibrations were performed using standard substrates and purified products. The substrate conversion rate at the thermodynamic equilibrium was obtained applying the following equation:

$$r(\%) = \left(1 - \frac{[\text{Substrate}]_{\text{equilibrium}}}{[\text{Substrate}]_{\text{initial}}}\right) \times 100. \quad (1)$$

Initial reaction rates were calculated by derivation of a second order polynomial approximation of the concentration profile, built on the five first experimental data (Software: Matlab®, MathWorks, USA).

2.4. Study of the enzymatic stability in the course of the reaction

The biocatalyst was incubated in 2-methyl-2-butanol in Wheaton® reactor at 55 °C for pre-defined durations (0, 2, 6, 10, 24 and 48 h). After this time, the substrates (6-amino-1-hexanol 0.12 M and ethyl oleate 0.24 M) previously solubilised were added to the reactor. The reaction was performed over 1 h. The initial consumption rate of ethyl oleate was assessed for each incubation time.

To study the protective effect of 6-amino-1-hexanol on the enzymatic stability in the 2-methyl-2-butanol, the biocatalyst was incubated in this solvent in Wheaton® reactor at 55 °C for 10 h, in presence of 0.12 M of 6-amino-1-hexanol. After this time, ethyl oleate 0.24 M was added to the reactor and the reaction was performed over 1 h. The initial rate of ethyl oleate consumption was then quantified and compared to the initial rate obtained in the case of 10 h of incubation without 6-amino-1-hexanol (control).

The residual enzymatic activity (A_r) was expressed as the ratio of the initial rate of ethyl oleate disappearance after a period t of incubation (A_t) over the initial rate of ethyl oleate disappearance without incubation (A_0):

$$A_r = \frac{A_t}{A_0} \quad (2)$$

2.5. Tool for model development

Following experimental investigations, several models based on classical mechanisms were studied. Seven differential equations describing mass balances were solved using the numerical integration tools present in the Matlab® software. Identification of the kinetic parameters was performed on each of the 10 experimental data sets. The optimisation tool was an innovative genetic-evolutionary algorithm developed in our laboratory. Its main features have been described in the literature [28,29]. The most suitable kinetic model is described in Section 3.

3. Results–discussion

The present work deals with the N,O-enzymatic trans-acylation of 6-amino-1-hexanol, a peptide-like model molecule. A previous study has shown that the Novozym 435®-catalysed acylation of 6-amino-1-hexanol (B) by an acyl donor (AR) in 2-methyl-2-butanol at 55 °C made it possible to obtain two acylated products. The first one was O-acyl aminohexanol (AB) and seemed to be rapidly converted into N,O-diacyl aminohexanol (ABA) thus suggesting a sequential reaction. The N,O-diacyl product was the major product at thermodynamic equilibrium [17]. Fig. 1 represents the assumed reaction scheme of this enzymatic acylation.

3.1. Preliminary tests

The first step consisted in evaluating the biocatalyst concentration which prevents enzyme concentration limitation in the course of the reaction. A convenient Novozym 435® concentration of 10 g L^{-1} was defined to prevent such limitation (data not shown). Under non-limiting biocatalyst concentration conditions, enzymatic trans-acylations of 6-amino-1-hexanol were performed with a quantitative monitoring of each substrate and product. The time profiles of the main reactants and products concentrations show that the kinetics of the reaction can be divided into two parts (Fig. 2). The first part corresponds to a fast reaction which takes place during the first hour. During this first period, the two substrates were rapidly converted with initial apparent rates of consumption of 6-amino-1-hexanol and ethyl oleate of 0.40 ± 0.02 M h^{-1}, and 0.55 ± 0.02 M h^{-1} respectively (corresponding to 0.67 ± 0.03 μmol min^{-1} per mg of biocatalyst, and 0.92 ± 0.03 μmol min^{-1} mg^{-1} respectively). The synthesis of both O-oleyl and N,O-dioleyl products was observed. In this