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Characterisation of pancreatic lipase substrate specificity in organic reaction media by a kinetic method

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Abstract

The possibility of assaying porcine pancreatic lipase specificity in organic media by kinetic modelling of esterification and transesterification reactions has been investigated. The calculated rate constant of the two-substrate reversible model was used as to estimate the chain length specificity in esterification reactions of fatty acids. This simple method is suitable to quantify the specificity in given reaction conditions, needing one single measurement for each substrate.

Keywords: Porcine pancreatic lipase; Substrate specificity; Esterification kinetics; Transesterification kinetics

1. Introduction

Esterification and transesterification reactions catalysed by lipases are among the most promising biotransformation processes [1]. Lipase activity and specificity in nonaqueous systems is dependent on a series of reaction parameters, which are difficult to control [2].

The substrate specificity of lipases has been intensively investigated in both biphasic reaction systems and organic reaction media. Kinetic models usually based on the application of Michaelis–Menten assumptions have been proposed, as those employing a competition factor to express substrate specificity [3–5].

The validity of any predictive model is generally limited to a particular type of reaction or enzyme. Therefore, is important to have the simplest possible method to determine the specificity for a given reaction. Usually, specificity can be quantified by kinetic parameters like the specificity constant (V_{max}/K_m) based on the measurement of initial reaction rates, but it needs series of measurements at different initial substrate concentrations [6].

The kinetic analysis of enzymatic esterification and transesterification reactions involving two substrates is much more difficult, but apparent specificity constants can be useful to characterise such systems [2]. Ping-pong [7,8]and ordered bi-bi [9] mechanisms have been proposed.

An alternative is to get kinetic parameters from a single progress curve. Such a kinetic model was originally elaborated by Kosugi and Suzuki [10] for the triglyceride hydrolysis reactions and extended by Han

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et al. [11] for hydrolysis reactions in reversed micelles, without large water excess. This second-order reversible model was used for the prediction of fractional conversion at a given reaction time.

In this work, the capability of the above-mentioned model for determination of the substrate specificity of pancreatic lipase in esterification and transesterification equilibrium reactions in organic media was investigated. Since the specificity is defined as a comparative difference in rates of catalysis, the kinetic rate constant of the direct reaction can be considered a measure of substrate specificity [12]. Fatty acid ester syntheses by direct esterification with 1-pentanol and acylation of diols by alkyl acetates were selected as model reaction systems.

2. Experimental

2.1. Esterification

The esterification reactions have been carried out using porcine pancreatic lipase preparations, obtained in our laboratory. The crude pancreatic powder was extracted with 0.1 M Tris–HCl buffer at pH 9.0 and precipitated with cold acetone, as previously described [13]. The water content of the lipase preparation was 3 wt.%. The water content of the initial reaction mixture was also determined by Karl Fisher titration and was set at 0.07 vol.% by adding the appropriate amount of distilled water. No water removal was carried out during the reaction.

In the general reaction procedure, equimolar amounts of 1-pentanol and fatty acid have been used. The initial concentrations were 0.15 M and *n*-hexane was employed as organic reaction medium. The experiments were accomplished at 37 °C in a 50 ml capacity thermostated glass reactor, with glass cap and magnetic stirring. The same stirring rate (250 rpm) was used in all experiments to avoid external mass transfer limitations.

The lipase preparation (45 mg mmol^{-1} fatty acid) was added to the reaction mixture at zero reaction time, and incubated for 24 h. At regular intervals of time, samples were taken, filtered to remove the enzyme and analysed by temperature programming in a Dani 86.10 gas chromatograph, equipped with a BPX-5 capillary column, 15 m × 0.32 mm (Scientific Glass Engineering), and flame ionisation detector. *n*-Decane (99.5%) from Merck was used as internal standard for the quantitative analysis.

High purity *n*-hexane (99%), obtained from Merck, was dried over 4 Å (8–12 mesh) molecular sieves, from Acros Organics. 1-Pentanol (99%) was from Fluka. The carboxylic acids: propionic, butyric, valeric (pentanoic), caproic (hexanoic), caprylic (octanoic), pelargonic (nonanoic), undecanoic, lauric (dodecanoic), myristic (tetradecanoic), palmitic (hexadecanoic), stearic (octadecanoic), and oleic (*cis*-9-octadecenoic), mainly from Merck, were used as purchased.

2.2. Transesterification

Essentially, the same experimental set-up described for esterification was employed to carry out the acylation reaction of diols having one or two secondary hydroxyl groups. Large excess of alkyl acetate (13:1 molar ratio related to the diol) was used as being in the same time the organic solvent for other components of the reaction system, except for the enzyme. The initial diol amount was 0.02 mol, and the same pancreatic lipase preparation has been used. The reaction time was 48 h and the temperature was maintained at 37 °C during the reaction. *n*-Hexadecane (99%) from Merck was utilised as reference substance for quantitative gas chromatographic analysis.

2-Ethyl-1,3-hexanediol and 2,5-hexanediol purchased from Fluka, 1,3-butanediol and 2,3-butanediol from Riedel de Haen have been used without further purification. Methyl acetate, ethyl acetate, *n*-propyl acetate, and *n*-butyl acetate from Chimopar (Romania) were anhydrified according to the literature data [14]. The initial water concentration of the reaction system was set at 0.07 vol.%, determined by the Karl Fisher method, using the same procedure as described for the esterification experiments.

2.3. Kinetic model

The kinetic model originally elaborated by Han et al. [11] obeys a two-substrate reversible reaction mechanism:

$$S_A + S_B \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} P_P + P_Q$$

where S_A and S_B denote the substrates (S_A being the limiting substrate), P_P and P_Q the products, and k_1

and k_{-1} are the rate constants of the direct and reverse process.

The rate expression according with this model can be represented by Eq. (1):

$$-\frac{dA}{dt} = k_1AB - k_{-1}PQ \quad (1)$$

where A and B are the concentrations of the substrates, P and Q the concentrations of the products at reaction time t.

We adapted this model to be used for esterification and transesterification reactions catalysed by lipases in organic reaction media.

The integrated rate equation corresponding to this mechanism results in the parametric Eq. (2), containing two parameters (X_E and ε) that can be determined by optimisation:

$$X = \frac{X_E(1 - e^{\varepsilon t})}{e^{\varepsilon t}[1 - \{1 + (A_0/B_0)\}X_E] + 1} \quad (2)$$

where A_0 and B_0 are the initial concentrations of the two substrates, X is the fractional conversion of the limiting substrate [$X = (A_0 - A)/A_0$], X_E the equilibrium conversion, t the reaction time, and ε a parameter related to the rate constant k_1, as results from Eq. (3). The rate constant of the direct reaction can be calculated using this equation:

$$k_1 = \frac{\varepsilon}{[A_0 + B_0 - (2B_0/X_E)]} \quad (3)$$

Optimisation by simplex method (a specially written program was employed for this purpose) gives the values of parameters X_E and ε that ensure the best fit of the model curve $X = f(t)$ resulted from Eq. (2) with the experimental data points.

Eq. (2) represents a kinetic model for reversible two-substrate enzymatic reactions, which does not take account of the consecutive intermediate steps involving binary or ternary complexes. Consequently, it is not accurate to describe the intimate reaction mechanism, but could be useful for specificity determination. Eq. (2) could also be used for the determination of the initial rates, as the first derivative at $t = 0$ gives the initial rate. The kinetic constant k_1 is appropriate to be considered a measure of enzyme specificity under given reaction conditions, requiring one single measurement for each substrate.

3. Results and discussion

3.1. Fatty acid chain specificity of porcine pancreatic lipase in esterification reactions

To perform this study, esterification reactions of saturated monocarboxylic acids by equimolar amounts of 1-pentanol have been carried out at 37 °C, using *n*-hexane as reaction medium. As shown in Fig. 1, the plots of the alcohol conversion vs. time resemble a Michaelis–Menten type saturation curve. The results presented in Fig. 1 were selected as examples of employing this kinetic model. It can be observed a good correlation between the kinetic model and experimental values. All the calculated kinetic rate constants of the investigated fatty acids are presented in Table 1.

The diagram from Fig. 2 indicates a high specificity of pancreatic lipase towards butyric and valeric acid, and a significantly lower esterification rate of acids with lower (propionic) and higher (C_6–C_{14}) carbon number. The specificity increased again for long chain fatty acids (palmitic and stearic), but remained significantly lower than observed for the best-suited substrates. For the same chain length, unsaturation leads to specificity decrease, as demonstrated for oleic acid compared to stearic acid. The acyl chain length specificity of this pancreatic lipase towards C_4–C_5 compounds is confirmed by our previous experiments involving triglyceride hydrolysis reactions [15]and is in agreement with results reported by other authors [4].

3.2. Kinetic modelling of acylation reactions of diols

As well as monohydroxyl alkyl alcohols, di- and polyols can be used as substrates for ester synthesis in lipase catalysed reactions. The main difficulty to accomplish these reactions consists in the low solubility of such substrates in nonpolar organic solvents commonly used as reaction media. A possibility to obtain diol esters by transesterification reactions with alkyl acetates was already demonstrated [16].

We used the acylation reaction of diols to demonstrate the validity of the kinetic model for transesterification reactions catalysed by pancreatic lipase. By using diols with a primary and a secondary hydroxyl group, the reaction was, as expected, regioselective

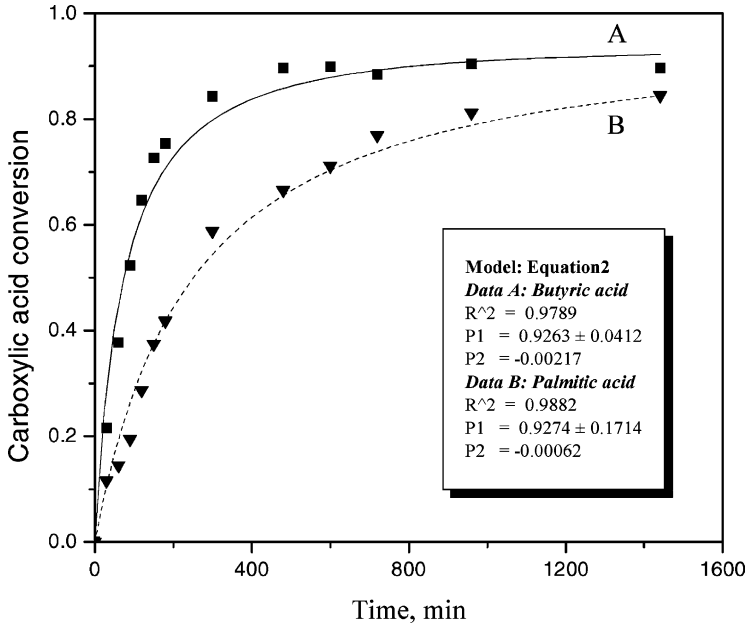

Fig. 1. Kinetic modelling of esterification reactions of butyric (curve A) and palmitic (curve B) acids by 1-pentanol catalysed by pancreatic lipase in *n*-hexane, optimised according to Eq. (2). The symbols are experimental data and the lines represent the values calculated according to Eq. (2).

for the primary esters. The selective synthesis of diol monoacetates is interesting as they can subjected further to other chemical or enzymatic reactions at the free OH group. This regioselectivity was based on kinetic discrimination, as diester formation was also observed at the final stage of the reaction. Supposing that the lipase amount is sufficient to avoid limitations due to the competition for the active centre, the reaction rate based on diol disappearance could be a measure of lipase specificity. Consequently, we used the kinetic rate constant k_1 for kinetic modelling of acylation reactions of diols.

Table 1
Kinetic rate constants of biocatalytic esterification reactions of fatty acids (0.15 M) by 1-pentanol (0.15 M) at 37 °C, catalysed by porcine pancreatic lipase

Carboxylic acid	Optimised parameters		Kinetic rate constant	
	X_E	ε	k_1 ($M^{-1}\,h^{-1}$)	Relative
Propionic (C_3)	0.911	−0.00016	0.32	0.06
Butyric (C_4)	0.926	−0.00217	5.57	1.00
Valeric (C_5)	0.957	−0.00165	7.38	1.32
Caproic (C_6)	0.968	−0.00026	1.56	0.28
Caprylic (C_8)	0.930	−0.00061	1.62	0.29
Pelargonic (C_9)	0.936	−0.00061	1.78	0.32
Undecanoic (C_{11})	0.888	−0.00073	1.16	0.21
Lauric (C_{12})	0.898	−0.00057	1.00	0.18
Myristic (C_{14})	0.933	−0.00038	1.06	0.19
Palmitic (C_{16})	0.927	−0.00062	1.58	0.28
Stearic (C18)	0.983	−0.00022	2.63	0.47
Oleic (C_{18}:C_1)	0.983	−0.00012	1.38	0.25

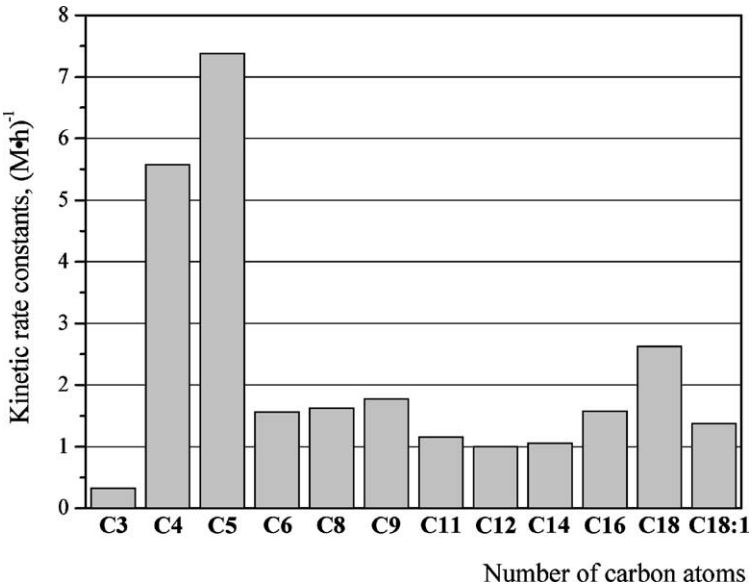

Fig. 2. Carboxylic acid specificity of pancreatic lipase in esterification reactions with 1-pentanol. The kinetic rate constants determined according to Eq. (3) and listed in Table 1 were plotted against the carbon atom number of the carboxylic acid.

A good agreement between the experimental data and the values of the substrate conversions calculated according to Eq. (2) was observed, as results from Fig. 3. The example presented in this figure shows the acylation reaction of 1,3-butanediol (2 g) by *n*-butyl acetate (38 ml), catalysed by porcine pancreatic lipase (1 g).

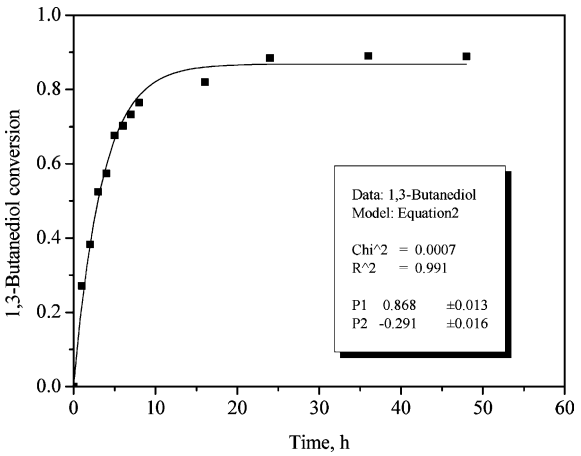

Fig. 3. Kinetic modelling of biocatalytic acylation reaction of 1,3-butanediol by *n*-butyl acetate, optimised according to Eq. (2).

No acylation reaction occurred with diols carrying only secondary hydroxyl groups as 2,3-butanediol and 2,5-hexanediol, indicating that the 1-monoester is a better substrate as the secondary diol (Table 2). Considering the four acylation reagents that have been tested, both 1,3-butanediol and 2-ethyl-1,3-hexanediol showed the highest rate constant when acylated by *n*-butyl acetate.

Table 2
Calculated kinetic rate constants of the biocatalytic acylation of diols with secondary hydroxyl group(s), catalysed by porcine pancreatic lipase

Diol	Acylation reagent	Kinetic rate constant, k_1 ($mM^{-1}\,h^{-1}$)
1,3-Butanediol	Methyl acetate	3.7
	Ethyl acetate	6.1
	n-Propyl acetate	12.9
	n-Butyl acetate	36.6
2-Ethyl-1,3-hexanediol	Ethyl acetate	6.6
	n-Propyl acetate	7.3
	n-Butyl acetate	9.4
2,3-Butanediol	*n*-Butyl acetate	–
2,5-Hexanediol	*n*-Butyl acetate	–

4. Conclusions

The substrate specificity of lipases in esterification reactions is important to obtain a better understanding of structure–function relationship and to improve the reaction parameters as a condition for the high conversions and better practical applicability.

Kinetic modelling of esterification and transesterification reactions catalysed by porcine pancreatic lipase in organic reaction was achieved using a method that needs a single progress curve for each substrate. The rate constants calculated by optimisation were used to quantify the chain length specificity of porcine pancreatic lipase in the esterification reaction of saturated monocarboxylic acids. This model could be also useful to measure the influence of the reaction conditions on the lipase specificity.

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