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► To cite this version:

Fabien Létisse, Sylvain Lamare, Marie-Dominique Legoy, Marianne Graber. Solid/gas biocatalysis: an appropriate tool to study the influence of organic components on kinetics of lipase catalyzed alcoholysis. *Biochimica et Biophysica Acta Proteins and Proteomics*, 2003, 1652, pp.27-34. hal-00329687

HAL Id: hal-00329687

<https://hal.science/hal-00329687>

Submitted on 13 Oct 2008

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Solid/gas biocatalysis: an appropriate tool to study the influence of organic components on kinetics of lipase catalyzed alcoholysis

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KEY WORDS

Lipase B from *Candida antarctica* – kinetics – transesterification – organic solvent – diffusionnal limitation – solid/gas biocatalysis

ABSTRACT

The influence of the addition of an extra component in a gaseous reaction medium, on the kinetics of alcoholysis of methyl propionate and *n*-propanol catalyzed by immobilized lipase B from *Candida antarctica* was studied in a continuous solid/gas reactor. In this reactor, the solid phase is composed of a packed enzymatic sample which is percolated by gaseous nitrogen, simultaneously carrying gaseous substrates and additional components to the enzyme while removing reaction products. The system permits to set thermodynamic activity of all gaseous components (substrates or not) independently at the desired values. This allows in particular to study the influence of an extra added component at a constant thermodynamic activity value, contrary to classical solid/liquid system, which involves large variations of thermodynamic activity of added solvent, when performing full kinetic studies. Alcohol inhibition constant (K_I) and methyl propionate and propanol dissociation constants (K_{MP} and K_P) have been determined in the solid/gas reactor in presence of 2-methyl-2-butanol, and compared with values previously obtained in absence of added component and in presence of water. Complementary experiments were carried out in the presence of an apolar compound (hexane) and led to the conclusion that the effect of added organic component on lipase catalyzed alcoholysis is related to their competitive inhibitory character towards first substrate methyl propionate. The comparison of data obtained in liquid or with gaseous 2-methyl-2-butanol shows that lower K_{MP} and K_I are found in gaseous medium, which would correspond on the one hand to a lower acylation rate k_2 , and on the other hand to a higher binding rate k_1 between substrate and free enzyme in gaseous medium.

1. INTRODUCTION

Biocatalysis in non-conventional media, especially in nearly anhydrous organic solvents, is today a topic of research which has been extensively explored [1,2]. These solvents possess great advantages such as shifting of thermodynamic equilibrium in favor of synthesis over hydrolysis, increased solubility of hydrophobic substrates and increased thermostability of the enzyme. Among the enzymes used in non-conventional media, lipases have drawn many attention [3,4]. The main interesting features of these enzymes are 1°) a number of lipases do show substantial activity in nearly anhydrous solvents; 2°) lipases tolerate great variation in experimental conditions in general, *i.e.* they remain stable and active; 3°) lipases catalyze a great number of different reactions including both regio- and enantio-selective synthesis.

As far as organic solvent effects on enzymatic catalysis are concerned, they have not been completely explained yet, even if some conclusions have been drawn :

Activity has been correlated with a number of solvent properties (hydrophobicity [5], polarizability [6], dielectric constant, viscosity [7]) of which the most important one is log P (the logarithm of the octanol-water partition coefficient), which is an indicator of solvent hydrophobicity. As catalytic activity usually increases with the amount of essential water in the vicinity of the enzyme, highly polar solvents, tending to strip away the layers of essential water from the enzyme by solubilizing large amounts of water, are causing inactivation. Conversely, hydrophobic solvents are less likely to cause inactivation.

In many studies it appears that solvent effects might be explained by an effect on the enzyme itself. Solvent effects have been ascribed to an effect on the conformational mobility of the enzyme [7], or to specific effects of the solvent on the enzyme, by binding of solvent molecules in the enzyme active site [8]. Other authors have suggested a non-specific interaction of the

solvent and the active site and have explained part of the solvent effects by competitive inhibition of the solvent [4,9].

As mentioned by many authors, the only way to assess the influence of the nature of the organic solvent on enzyme kinetic behavior is to perform a full kinetic description based on proper determination of V_M and K_M values in different media [10,11].

Besides, when a comparison of enzyme behavior in different solvents has to be made, the “availability of substrate to the enzyme” can not be quantified using its concentration mainly because substrate solvation is modified as the reaction medium changes [12,13]. One of the methods proposed for correcting about solvation of substrates and products, is the replacement of concentrations by thermodynamic activities in the rate equations [14]. To proceed such corrections, activity coefficients (γ -values) of substrates are needed; they are either determined experimentally or estimated by using the UNIFAC group contribution method [4,15].

When performing studies concerning the effect of solvent on kinetic parameters of enzymes, with correction for solvation of substrates, authors frequently call UNIFAC predictions into question, as sources of inaccurate γ -values, being the cause of differences in enzyme performance observed in various solvents [4,16,17]. Deviations in the UNIFAC calculations up to a factor of two have been reported [16], rendering correction for solvation of substrates far from being straightforward. Moreover, in most of the experiments, γ -values are determined for one set of substrates and solvent quantities and considered to be constant in the range of concentrations used. These approximations lead to potentially cumulative errors on kinetic constant determination.

In this paper, we used a continuous gas phase reactor to study the effect of organic molecules on kinetic parameters of the alcoholysis of methyl propionate by 1-propanol catalyzed by

immobilized lipase B from *Candida antarctica* (CALB). In this system a solid packed enzymatic sample is percolated by a carrier gas, which simultaneously carries gaseous substrates and if necessary an added gaseous component to the enzyme and removes reaction products. The gas phase reactor we used offers the possibility to control and adjust thermodynamic activities of reaction species, on the one hand, and of an extra added component on the other hand, by varying the partial pressure of each compound in the carrier gas. A complete explanation of the design of the reactor and of the theoretical background for thermodynamic activities control has been presented previously [18]. This technology, which already allowed to study the role of water on the alcoholysis reaction [19], thus appears as a powerful tool for the investigation of the effect of organic component on the activity of the enzyme.

2. EXPERIMENTAL

2.1. Enzyme and chemicals

Chirazyme L2 (Type B lipase from *Candida antarctica* lyophilized) was a gift from Roche Industrie (Penzberg, Germany).

All substrates were of the highest purity (99% minimum). Chemicals were dried prior to use. Propanol and 2-methyl-2-butanol commercial solutions were dried by refluxing with magnesium and then distilled. Methyl propionate commercial solution was dried with Na_2CO_3 and distilled from P_2O_5 and hexane commercial solution was dried by refluxing with P_2O_5 and then distilled from P_2O_5 . The dried solvents were stored under argon atmosphere and over molecular sieves.

2.2. Adsorption of lipase onto a solid support

Enzyme adsorption was performed onto Chromosorb P AW DMCS, mesh 60-80 (Acid Washed DiMethylChloroSilane) (Prolabo, France) as described in a previous work [20]. Average diameter

of the particles used was 177-255 μm , volume and diameter of pores were $1.6 \text{ cm}^3 \cdot \text{g}^{-1}$ and $5.4 \mu\text{m}$ respectively, density was $0.47 \text{ g} \cdot \text{cm}^{-3}$ and specific area was $4.0 \text{ m}^2 \cdot \text{g}^{-1}$.

2.3. Experimental setup for solid-gas catalysis

The bioreactor used in this study has already been described in a previous publication [19].

A typical experiment was run at 70°C , with 5 to 15 mg of adsorbed enzyme, 1 mg of adsorbed enzyme preparation corresponding to $3.32 \mu\text{g}$ of lyophilized lipase. The total flow passing through the reactor was set at $1 \text{ mmol} \cdot \text{min}^{-1}$ giving a volumetric flow of $28.5 \text{ mL} \cdot \text{min}^{-1}$. In these conditions, less than 20% of the substrates were converted allowing thus to assume that initial rates (expressed in μmoles of product formed per minute and per mg of enzymatic preparation) were measured and that inhibition by the products did not occur.

2.4. Chromatographic assays

The vapor phase leaving the bioreactor was sampled using a 0.25 mL loop on a six-way valve (Valco) maintained at 150°C . Samples were automatically injected in the split injector of a gas chromatograph (Hewlett Packard model 5890 A) equipped with a Flame Ionization Detector (FID) for detection of all products. The column used was an OV 01 ($30 \text{ m} \times 0.32 \text{ mm i.d.} \times 1 \mu\text{m}$ film thickness, Chrompack, France). The split ratio was 200:1. The injector and the detector were kept at 220°C and 250°C respectively. Carrier gas was nitrogen and the flow rate in the column was $1.1 \text{ mL} \cdot \text{min}^{-1}$. Hydrogen and air were supplied to the FID at 38 and $390 \text{ mL} \cdot \text{min}^{-1}$ respectively.

For the quantification of methanol, propanol, methyl propionate and propyl propionate, in the presence of 2-methyl-2-butanol, the initial column temperature was 35°C and it was increased to 80°C using two different rates: $0.5^\circ\text{C min}^{-1}$ from 35°C to 39°C and 5°C min^{-1} from 39°C to 80°C .

For the quantification of the substrates and products, in the presence of hexane, the column temperature was held at 33°C for 7.5 min, then programmed to increase at 7°C.min⁻¹ to 85°C.

Quantitative data were obtained after integration on a HP 3396A integrator. An external standard method was used for the calculations.

3. RESULTS

3.1 Kinetic studies in presence of organic compound or water at fixed thermodynamic activity in the solid/gas reactor

As demonstrated in previous works [19,20], the reaction of alcoholysis between methyl propionate and propanol catalyzed by CALB in the solid/gas reactor, in absence of any added component or in the presence of water, can be modeled by a Ping Pong Bi Bi mechanism with dead-end inhibition by the alcohol.

In this reaction sequence, the lipase reacts with methyl propionate to yield the lipase.....methyl propionate complex or with propanol to yield a dead-end enzyme.....propanol complex. Then the lipase..... methyl propionate complex is transformed into an acyl-enzyme intermediate and the first product (methanol) is released. The second substrate, propanol, next interacts with the acyl-enzyme to form another binary complex (Acyl-E..... P), which finally yields propyl propionate and free lipase (scheme 1).

Experimental kinetic data for the reaction of alcoholysis between methyl propionate and propanol catalyzed by CALB in the solid/gas reactor, in presence, on the one hand, of 2-methyl-2-butanol at a thermodynamic activity equal to 0.3 ($a_{2M2B}=0.3$), or in presence, on the other hand, of hexane at a thermodynamic activity equal to 0.3 ($a_{hexane}=0.3$), were plotted and analyzed with the method explained below:

The kinetic rate expression for the Ping Pong Bi Bi mechanism of the lipase, considering that the experiments were performed under conditions in which the influence of the products can be neglected, can be written as shown in Equation (1).

$$v_i = \frac{V_{Mapp} \cdot a_{MP}}{K_{Mapp} + a_{MP}} \quad (1)$$

$$\text{with } \begin{cases} \frac{K_{Mapp}}{V_{Mapp}} = \frac{K_{MP}}{V_M} \left(1 + \frac{a_P}{K_I} \right) & (2) \\ V_{Mapp} = \frac{a_P \cdot V_M}{K_P + a_P} \Leftrightarrow V_{Mapp} = -\left(K_P \cdot V_{Mapp} / a_P \right) + V_M & (3) \\ K_{Mapp} = K_{MP} \cdot \frac{a_P \cdot (1 + a_P / K_I)}{K_P + a_P} & (4) \end{cases} \quad \text{and } \begin{cases} K_{MP} = \frac{k_{-1} + k_2}{k_1} & (5) \\ K_P = \frac{k'_{-1} + k'_2}{k'_1} & (6) \end{cases}$$

E = enzyme, MP = methyl propionate, P = propanol, M = methanol, PP = propyl propionate
 a_P and a_{MP} are thermodynamic activities of propanol and methyl propionate respectively and K_I , K_{MP} and K_P are the dissociation constants of the propanol..... enzyme, methyl propionate..... enzyme and acyl-enzymepropanol complexes respectively.

The determination of dissociation constants (K_I , K_{MP} and K_P) was performed according to the methodology explained in details in a previous publication [20].

As an example figures 1 to 3 show the treatment of the results obtained in presence of 2-methyl-2-butanol.

Both sets of experiments obtained in presence of 2-methyl-2-butanol or hexane, showed that the kinetics agreed with the Ping-Pong Bi Bi mechanism with dead-end inhibition by the second substrate propanol.

3.2 Inhibition studies using different thermodynamic activities of 2-methyl-2-butanol, hexane and water

Results obtained for the dissociation constant of the propanol.....enzyme complex, K_I , are summarized in Table 1, with results previously obtained without added component and in presence of water [19,20]. The variation of K_I , with the nature of component added at a thermodynamic activity equal to 0.3 shows that inhibition by propanol is stronger (K_I lower) as the hydrophobicity of the added component increases (K_I hexane < K_I 2-methyl-2-butanol < K_I water).

In the next set of experiments, 15 mg of supported enzyme was placed in the solid-gas bioreactor and percolated with nitrogen together with the two substrates and 2-methyl-2-butanol or hexane. Methyl propionate and 1-propanol thermodynamic activities were kept constant (0.15) while thermodynamic activity of added components was scanned from 0 to 0.6.

On Figure 4 the effect of a_{2M2B} , a_{hexane} and a_w on the catalytic rate is shown. For these three components, the initial velocity decreases when their thermodynamic activity increases, indicating the inhibitory character of these components. In a previous study [20], it was shown that water was a competitive inhibitor of methyl propionate, as propanol is, with an inhibition constant 5 times higher than the one of propanol. It appears on figure 4 that, for thermodynamic activities superior to 0.2, the inhibitory character of the added components decreases when their hydrophobicity increases.

At a thermodynamic activity equal to 0.1, 2-methyl-2-butanol departs from this rule, by inhibiting more strongly lipase activity than water. This has to be related to the general water-induced enzyme activation, observed when biocatalysis is performed in anhydrous medium. It was shown that this role is predominant at $a_w=0.1$ and that optimal hydration level is reached for $a_w > 0.1$ [19,21].

3.3 Comparison between propanol inhibition constant and enzyme-substrate dissociation constants in presence of 2-methyl-2-butanol in the solid/gas reactor and in liquid medium

On inspecting the values of propanol inhibition constant and enzyme-substrate dissociation constants in presence of 2-methyl-2-butanol in the solid/gas reactor and in liquid medium (Table 2), it appears that values of K_I and K_{MP} are respectively 3.5 and 6 times higher in liquid medium than in the solid/gas reactor. As far as K_P is concerned, similar values are obtained in gaseous and liquid media.

It has to be noticed that kinetic parameters of alcoholysis of methyl propionate and *n*-propanol in presence of 2-methyl-2-butanol obtained in the gas/solid reactor, are determined at constant a_{2M2B} (*i.e.* 0.3), by fixing the partial pressure of added 2-methyl-2-butanol in the carrier gas at the adequate value, and varying a_{MP} and a_P to perform the full kinetic study. On the contrary, for the determination of kinetic parameters in liquid 2-methyl-2-butanol, the full kinetic study presented in a previous study [20], involves variations of a_{2M2B} between 0.33 and 0.96. These a_{2M2B} values are obtained by calculations, from molar fractions using the UNIFAC group contribution method, and correspond for $a_{2M2B} = 0.33$ to the highest substrate quantities used ($a_P = 0.5$ and $a_{MP} = 0.3$) and for $a_{2M2B} = 0.96$ to the lowest substrate quantities used ($a_P = 0.01$ and $a_{MP} = 0.05$). Indeed the added 2-methyl-2-butanol constitutes in this case the reaction medium and the solvent of the substrates.

4. DISCUSSION

4.1 Effect of organic added component in the solid/gas reactor

Whatever the added molecule (water, 2-methyl-2-butanol or hexane) in the system is, the reaction mechanism followed by immobilized CALB in the solid/gas reactor remains a Ping Pong Bi Bi

mechanism with dead-end inhibition by the alcohol. This result is in accordance with numerous studies on kinetics of acyl transfer reactions in organic media catalyzed by this enzyme, in which the mechanism of action was similar in different solvents [9,22,23].

On the contrary the values of kinetic constants were found to vary with the type of added component. For the determination of these values, the availability of the different chemical species in the gas phase for the enzyme, were quantified by using their thermodynamic activities. The significance of using this parameter has already been discussed in previous publication [18].

The use of thermodynamic activity has already proved to considerably lower the differences between kinetic parameters values obtained in different liquid organic solvents, as it permits to correct substrates for solvation effects [16,22,23]. As mentioned previously, the solid/gas reactor allows control and independent variations of thermodynamic activities for the different reaction species, by varying the partial pressure of each compound in the carrier gas. Problems encountered in studies performed in liquid organic media, to evaluate differences in solvation of the substrates, in particular in determining activity coefficients, are therefore avoided.

Moreover, in the solid/gas reactor, when an extra component is added in the gaseous feed, in addition to substrates, its thermodynamic activity can be fixed to a constant value, while simultaneously varying the quantity of delivered substrates. The effect of this added component on enzyme kinetics, can therefore be better assessed. This constitutes a significant advantage over classical solid/organic liquid system, since, in practice, totally inert solvents do not exist and because full kinetic study performed in liquid medium, including activity measurements over a certain substrate concentration range, involves very large variations of solvent thermodynamic activities.

For these reasons, kinetic parameters obtained in the solid/gas reactor can really be considered as “intrinsic kinetic parameters” in presence of different added components at fixed thermodynamic activity.

In this way, differences between intrinsic parameters quoted in tables 1 and 2 indicate that organic components interfere with the binding process between substrate and immobilized enzyme. In several studies performed in organic and gaseous media, solvent or gaseous added component effects on the enzyme, have been ascribed to specific effects of these components on the enzyme, *e.g.* by binding of molecules to the enzyme active site [21,24,25]. As a result, such molecules that may have been lying around the active site have to be displaced before the substrate gets in. This renders the energy of desolvation or “component release” of the active site dependent of the nature of the added solvent or gaseous. Another assumption is that the bound substrate is not completely shielded from the solvent. Although no data are available for lipase, for acyl-subtilisin complex in the ground state it was shown that approximately one third of the substrate is exposed to the solvent in liquid medium [24].

Another possibility is that the gaseous added component is acting as a competitive inhibitor. In solid/liquid systems, competitive inhibition by solvents has been mentioned by many authors, as a possible explanation of the effect of solvents [4,9,22]. Non-specific interactions of the solvent and the active site are generally suggested, to explain the increase of K_m and K_I values of the substrates promoted by solvents. To better define and explain such effect in the case of CALB catalyzed alcoholysis kinetics, authors included an initial hypothetical equilibrium step between enzyme and solvent, in the kinetic model [23]. In an other study, the same authors observed that alcohol inhibition and enzyme-substrate dissociation constants were lower in presence of hydrophobic solvents, whereas hydrophilic solvents tend to induce higher values of these parameters. This means that with hydrophobic solvents, inhibition and substrate binding to the

active site is stronger, than in presence of hydrophilic ones. To explain these results, the main assumption was that solvents are acting as competitive inhibitor [22].

In our case, the inhibitory effect of gaseous hexane, 2-methyl-2-butanol and water is clearly demonstrated in the solid/gas reactor, by studying the effect of increasing thermodynamic activities of these components on lipase activity (figure 4). As mentioned in the “results” part, the inhibitory character of the added components decreases when their hydrophobicity increases. This inhibitory effect has to be related to the effect of these components on the intrinsic value of K_I , the dissociation constant of propanol..... enzyme complex (table 1): K_I decreases when the hydrophobicity of added component increases, which means that the inhibition by propanol is stronger in presence of hydrophobic component like hexane.

It is thus clearly demonstrated in this study that the effect of added organic component on alcoholysis catalyzed by CALB is related to their inhibitory character. As reported by Uppenberg *et al.* [26], the region around the catalytic serine (Ser-105) is polar in nature so hydrophilic components may bind to this region to form a dead-end complex. This explains the differences of the K_I intrinsic values obtained in presence of an apolar component like hexane, of a moderately polar tertiary alcohol such as 2-methyl-2-butanol and in presence of water.

4.2 Comparison of lipase kinetic behavior in gaseous and liquid media

Table 2 indicates that values of K_I and K_{MP} obtained for alcoholysis of methyl propionate and *n*-propanol catalyzed by immobilized CALB are respectively 3.5 and 6 times higher in liquid 2-methyl-2-butanol than in the solid/gas reactor in presence of gaseous 2-methyl-2-butanol.

The first assumption is that kinetic constants obtained in liquid 2-methyl-2-butanol are overestimated owing to diffusional limitations affecting substrate access to the enzyme molecules. Such limitations occurring in liquid medium and disappearing in gas, could explain

the different kinetic behavior between the two media. As far as external diffusion is concerned, it can be assessed that the experimental conditions (high agitation rate, small diameter of particles), allow to overcome such limitations. On the contrary, as enzyme used in this study was immobilized into a porous support, the possible occurrence of internal diffusion has to be studied. To investigate the importance of internal diffusion, the reaction and diffusion rates into the porous particle of immobilized enzyme have to be compared. The modified Thiele's modulus (ϕ_m) defined by Weisz and Prater [27], which corresponds to the ratio between the both rates, has thus been used. It is defined as follow:

$$\phi_m = \frac{R^2}{D_s^{eff} \times C_s} \times \left(\frac{-1}{V_c} \times \frac{dn}{dt} \right) \quad (7)$$

Where R is the radius of the particle (cm), and V_c is the catalyst volume (dm^3). C_s is the concentration at the surface of the particle, considered as equal to the concentration in bulk organic phase ($\text{mol} \cdot \text{dm}^{-3}$). In order to display the potential influence of internal diffusion on the kinetic behavior of the enzyme, the concentrations of substrates chosen for Thiele's modulus calculus have to ensure the maximal reaction rate. The highest measured rate was $9.80 \times 10^{-5} \text{ } \mu\text{mol} \cdot \text{s}^{-1}$ for methyl propionate and propanol thermodynamic activities of 0.3 and 0.1 respectively, corresponding to a methyl propionate concentration of $1.99 \text{ mol} \cdot \text{dm}^{-3}$ and a propanol concentration of $0.90 \text{ mol} \cdot \text{dm}^{-3}$. D_s^{eff} is the effective diffusivity of the substrate in the porous support ($\text{cm}^2 \cdot \text{s}^{-1}$) and is related to the substrate diffusivity in the bulk solution (D_s) by the following expression [28]:

$$D_s^{eff} = \frac{\varepsilon \times D_s}{\tau} \quad (8)$$

Where ε is the void fraction in the porous catalyst (0.752 for Chromosorb P AW-DMCS) and τ the tortuosity factor that takes into account the pore geometry. Its value is usually taken between

1.5 and 5 ($\tau = 3.25$ as an average value). The substrate diffusivity in the organic solvent can be estimated from the Shiebel relation [29]:

$$D_s = k \times \frac{T}{\eta_B \times V_S^{1/3}} \quad (9)$$

Where k is a constant and is equal to 17.5×10^{-8} , η_B the viscosity of the solvent (2-methyl-2-butanol) (cP), V_S the molar volume of the substrate at its normal boiling point ($\text{cm}^3 \cdot \text{g}^{-1} \cdot \text{mol}^{-1}$) and T the temperature (K).

Weisz and Prater [27] have shown that diffusional effects become significant for $\phi_m > 1$; for $\phi_m < 1$ the reaction is essentially kinetically controlled. Calculus of ϕ_m for methyl propionate and propanol was performed using values for D_s^{eff} estimated by the procedure described above, values for ϕ_m of 0.021 and 0.062 were obtained respectively. Thus, it is possible to state that internal diffusion resistances play no significant role for both substrates in liquid media using 2-methyl-2-butanol as solvent.

Another simple assumption which could explain part of the differences of K_I and K_{MP} values obtained using the solid/gas reactor and liquid medium is the difference of 2-methyl-2-butanol availability in the two media. As mentioned above, full kinetic study of alcoholysis performed in liquid 2-methyl-2-butanol involves variations of a_{2M2B} between 0.33 and 0.96, whereas kinetic parameters of this reaction in the solid/gas reactor are obtained at constant $a_{2M2B}=0.3$. Therefore a_{2M2B} used in liquid medium is on average higher than in the solid/gas reactor, which leads higher values of K_I and K_{MP} in liquid medium, because of the inhibitory character of 2-methyl-2-butanol. Furthermore variations of a_{2M2B} used in liquid medium induce errors in kinetic parameters determination. This fact, which is never noticed in studies concerning the influence of

the nature of organic solvent on enzyme kinetic parameters, should be kept in mind to avoid misinterpretations.

Finally, the effect of the state of reaction medium (liquid or gaseous) on these parameters, has to be related to the differences between energy of desolvation of the active site filled with liquid 2-methyl-2-butanol and energy of gaseous 2-methyl-2-butanol release from the active site. Indeed 2-methyl-2-butanol has to be displaced before the alcohol inhibitor or the first substrate methyl propionate binds with enzyme's active site [19]. The energy of this displacement depends on the quantities of bound molecules and on the energetic level of one bond, (which both vary with the state of the reaction medium). As far as the variation of K_{MP} is concerned, a second effect of the state of reaction medium on acylation rate k_2 has to be considered, as according to equation (5),

$$K_{MP} = \frac{k_{-1} + k_2}{k_1}$$
. This additional effect would explain the more pronounced difference between

K_{MP} in the liquid and gaseous media than the difference obtained for K_I .

Consequently the lower K_{MP} in gaseous medium in presence of gaseous 2-methyl-2-butanol would correspond on the one hand to a lower acylation rate k_2 in this medium than in liquid 2-methyl-2-butanol, and on the other hand to a higher binding rate k_1 between substrate and free enzyme. Future work will concentrate on these assumptions.

Finally results obtained in the present work about the influence of organic compounds on kinetics of alcoholysis catalyzed by lipase in a solid/gas reactor, and results previously obtained concerning the effect of water [19] lead to the following conclusion : the dissociation constant between enzyme and acyl substrate (K_{MP}) and the alcohol inhibition constant (K_I) are strongly enhanced by a polar compound like water, and fairly enhanced by a moderately polar compound like 2-methyl-2-butanol. On the contrary, an apolar compound like hexane has no effect on K_I (at

$a_{\text{hexane}}=0.3$). These effects have to be related to the more or less pronounced competitive inhibitory character of the compounds, arising from interactions with polar region around the catalytic site of the enzyme.

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FIGURE LEGEND

Figure 1: Reciprocal initial rates versus reciprocal a_{MP} for different a_P values in presence of 2-methyl-2-butanol.

Reaction was carried out in the solid/gas bioreactor at 70°C, at a molar flow of 1 mmol.min⁻¹ in the presence of 5 to 15 mg of adsorbed CALB onto Chromosorb AW DMCS. 2-methyl-2-butanol thermodynamic activity was set to 0.3.

Figure 2: Determination of the dissociation constant of the enzyme..... propanol complex (K_I) in the solid/gas bioreactor in presence of 2-methyl-2-butanol at a thermodynamic activity equal to 0.3.

Figure 3: Determination of K_P and K_{MP} in presence of 2-methyl-2-butanol at a thermodynamic activity equal to 0.3. in the solid/gas bioreactor. Further explanation for the determination of these parameters is extensively detailed in a previous publication [20].

Figure 4: Effect of water, hexane and 2-methyl-2-butanol thermodynamic activity on initial rates.

Reaction was carried out at 70°C, at a molar flow of 1 mmol.min⁻¹ in the presence of 15 mg of adsorbed CALB onto Chromosorb AW DMCS. Methyl propionate and propanol thermodynamic activities were fixed at 0.15 and thermodynamic activity of solvents was varied from 0 to 0.6.

Table 1 : Effect of water, 2-methyl-2-butanol and hexane on dissociation constants of propanol..... enzyme complex (K_I) in the solid/gas reactor

Added component	Thermodynamic activity	K_I
None	0	0.019*
Water	0.3	0.161**
2-methyl-2-butanol	0.3	0.045
Hexane	0.3	0.018

* results from Bousquet-Dubouch *et al.* (2001)

** results from Graber *et al.* (2003a)

Table 2 : Dissociation constants of propanol enzyme, methyl propionate enzyme and acyl-enzyme..... propanol complexes respectively (K_I , K_{MP} and K_P) in presence of 2-methyl-2-propanol in the solid/gas reactor and in liquid medium.

	a_{2M2B}	K_I	K_{MP}	K_P
Solid/gas reactor	0.3	0.045	0.106	0.023
Liquid medium*	0.33 to 0.96	0.160	0.651	0.020

* results from Bousquet-Dubouch *et al.* (2001). Since all concentrations in liquid medium are linked together, data were obtained under the following range of experimental conditions: $a_{2M2B} = 0.33$ ($a_p = 0.5$ and $a_{MP} = 0.3$), and $a_{2M2B} = 0.96$ ($a_p = 0.01$ and $a_{MP} = 0.05$)

FIGURE 1

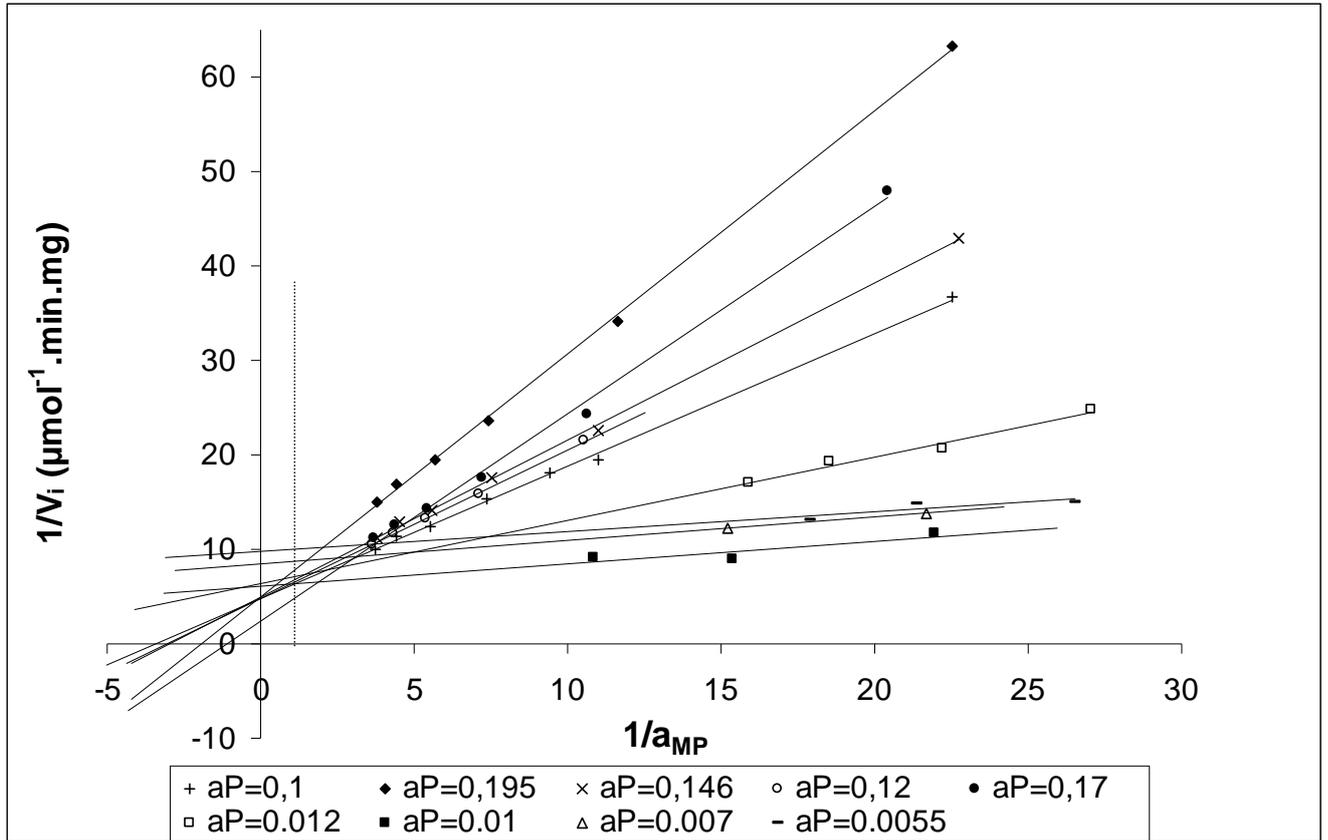


FIGURE 2

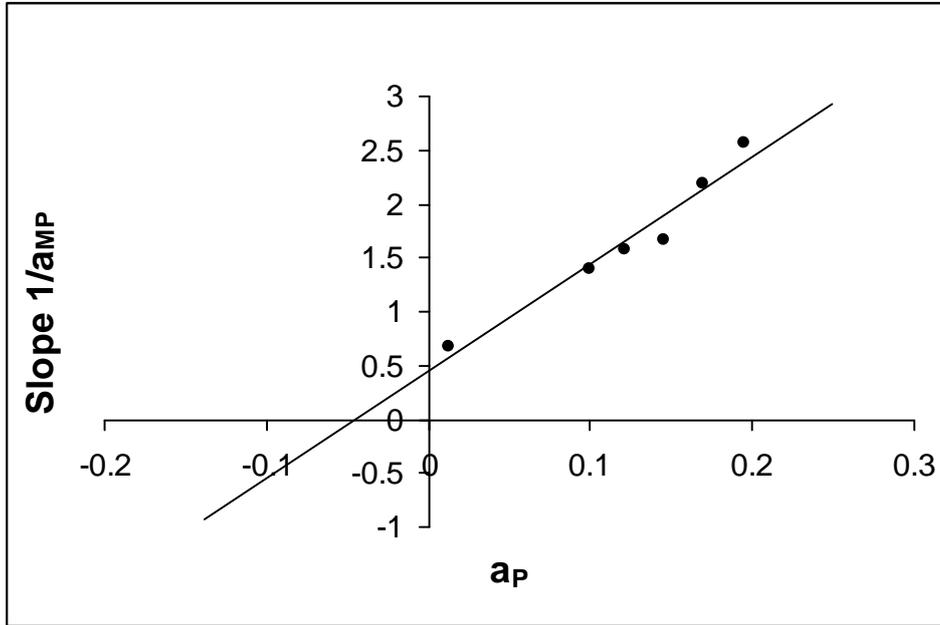


FIGURE 3

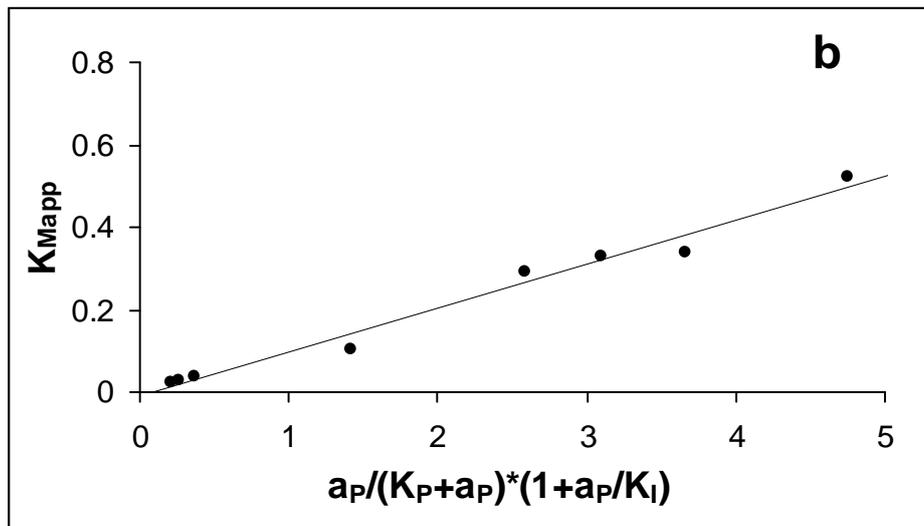
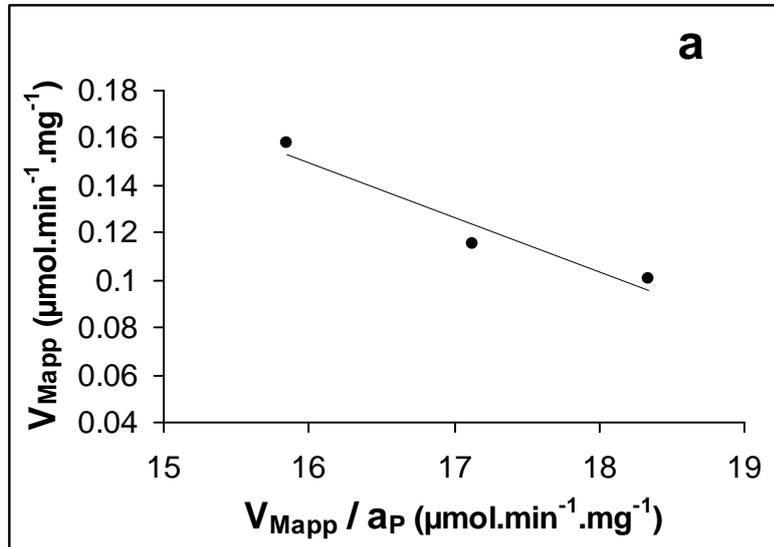


FIGURE 4

