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KEY WORDS

Lipase B from *Candida antarctica* – Kinetics – transesterification – competitive inhibition – Ping Pong Bi Bi mechanism

ABSTRACT

The kinetics of alcoholysis of methylpropionate and n-propanol catalyzed by *Candida antarctica* lipase B supported onto silanized Chromosorb P was studied in a continuous solid/gas reactor. In this system the solid phase is composed of a packed enzymatic sample and is percolated by nitrogen as carrier gas, which simultaneously carries substrates to the enzyme while removing reaction products. In this reactor the thermodynamic activity of substrates and effectors can be perfectly adjusted allowing kinetics studies to be performed under different operating conditions. The kinetics obtained for alcoholysis were suggested to fit a Ping Pong Bi Bi mechanism with dead-end inhibition by the alcohol. The values of all apparent kinetic parameters were calculated and the apparent dissociation constant of enzyme for gaseous ester was found very low compared with the one obtained for liquid ester in organic medium, certainly due to the more efficient diffusion in the gaseous phase. The effect of water thermodynamic activity was also investigated. Water was found to act as a competitive inhibitor, with a higher inhibition constant than n-propanol. Thus alcoholysis of gaseous methylpropionate and n-propanol catalyzed by of *Candida antarctica* lipase B was found to obey the same kinetics mechanism than in other non conventional media such as organic liquid media and supercritical carbon dioxide, but with much higher affinity for the substrates.

INTRODUCTION

Biocatalysis in non-conventional media, especially in organic solvents, is today a topic of research which has been extensively explored. Organic solvents possess great advantages when synthesis needs to be performed without water and/or when substrates and/or products are insoluble in aqueous media.

Kinetics studies using lipases as catalysts in such solvents have only become important in the last few years. Chulalaksananukul et al. [1] first proposed a model based on the Ping Pong Bi Bi mechanism for the kinetics of esterification of oleic acid with ethanol using immobilized *R. miehei* lipase as biocatalyst. Then, several studies confirmed that experimental data for esterification, alcoholysis and ester exchange reactions catalyzed by lipases in various organic solvents were well fitted by this model [2, 3, 4, 5, 6, 7, 8]. In most cases, competitive inhibition by the alcohol was reported. However, in esterification reactions, water is one of the products and is usually present in the reaction medium since some water is essential for enzyme activity. Therefore, the Ping Pong Bi Bi model, which is based on initial rate conditions with negligible product levels, does not seem to be appropriate. As a consequence, Janssen et al. [9] recently suggested a revised model for esterification reactions. They included an extra parameter which take into account the presence of water. Nevertheless, for alcoholysis reaction where water is neither a substrate nor a product of the reaction, the Ping Pong Bi Bi model seems to be unanimously accepted, since hydrolysis reaction is negligible. Several studies run in supercritical carbon dioxide also showed that experimental data were in good agreement with this model [10, 11, 12].

Concerning the study of enzyme kinetics in solid/gas, the only available data have been reported by Lamare and Legoy [13]. In this work, immobilized *Fusarium solani* cutinase was used to catalyze alcoholysis on short volatile esters in continuous solid/gas reactor. The

enzyme was found to have an unusual kinetic behavior since a sigmoid relationship between the rate of alcoholysis and the thermodynamic activity of the acyl-substrate was observed. Recently, the same enzyme immobilized on zeolite was found to obey a classical Ping Pong Bi Bi kinetic model when run in isooctane [14]. Therefore, further studies are needed to determine whether such differences in kinetics behavior between liquid/solid and gas/solid systems can be extended to other enzymes.

As reported by many authors, substrate availability in non-conventional media can not be quantified using its concentration mainly because substrate solvation is modified as the reaction medium changes [15, 16]. Different methods have been proposed for correcting for solvation of substrates and products. All of these methods involve the choice of a reference or standard state [17, 18]. Corrections based on a transfer energy method [8, 17, 19, 20] or on the use of partition coefficients to correct the Michaelis constant [18] have been reported. A third method is to express the kinetic constant in terms of thermodynamic activities instead of concentrations [18, 21, 22]. However, in all cases where organic solvents are used, data such as activity coefficients or partition coefficients are needed to proceed such corrections; they are either found in the literature or determined experimentally. Another frequently used alternative is to estimate activity coefficients by using the UNIFAC group contribution method [23].

So, despite the large amount of successful work completed in organic solvents, it is still difficult to establish general rules related to their use. Enzyme specificity cannot be predicted from solvation data only and deviations have been observed between corrected kinetic parameters depending on the chosen standard state. Therefore, experimental data are necessary for every substrate [21].

Contrary to the classical solid/organic liquid system, the solid/gas process offers the possibility to perfectly control and adjust thermodynamic activities of reaction species. On a

fundamental point of view, this technology thus appears as a powerful tool for the investigation of the influence on catalysis of enzyme microenvironment. Moreover, in this system, the study is limited to the enzyme and the substrates/products/water since no species are predominant (contrary to synthesis where a solvent is present). As a consequence, solvation is fully controlled.

On an applied point of view, solid/gas biocatalysis presents numerous advantages over conventional solid/liquid system [24].

In this paper, the solid/gas system was used for the alcoholysis of methylpropionate and 1-propanol using immobilized *Candida antarctica* lipase B (CALB) as biocatalyst. This lipase has already been successfully used as biocatalyst for the synthesis of various esters in organic media [8, 25, 26]. Moreover, its crystal structure has been determined and it was showed that the lipase has a catalytic triad, similar to the one found in other lipases, and also reveals a solvent accessible active site [27, 28]. The alcoholysis reaction catalyzed by this lipase in organic media follows a Ping Pong Bi Bi mechanism, with a competitive substrate inhibition by the alcohol [8]. So, the purpose of this work was to study the kinetics of a classic alcoholysis reaction to determine whether this lipase behaves differently when substrates are gaseous and when no predominant species (no solvent) are responsible for solvation of enzyme and substrates. This could give precious information on the molecular mechanism of this enzyme.

EXPERIMENTAL

Enzyme and chemicals

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

Methylpropionate and propylpropionate were purchased from Fluka (S^{t}Quentin-Fallavier, Switzerland). Propionic acid was from Sigma (St Louis, MO). All the substrates were of the highest purity (99% minimum) and checked by gas chromatography before use. Chemicals were dried over molecular sieves and filtered just before use. Pure water was obtained via a Milli Q system (Millipore, France).

Adsorption of lipase onto a solid support

Enzyme adsorption was performed onto Chromosorb P AW DMCS), mesh 60-80 (Acid Washed DiMethylChloroSilane) (Prolabo, France). In a typical adsorption procedure, 10 mg of enzyme was dissolved in 6 mL phosphate buffer, pH 7.5, 20 mM, and 3 g of dry Chromosorb P AW DMCS was added to the solution. After vigorous shaking, the preparation was left one week under vacuum and over P_2O_5. The dry Chromosorb loaded with enzyme was then stored at 4°C over silica gel.

Experimental setup for solid-gas catalysis

The bioreactor used in this study has already been described in a previous publication [24]. The packed bed bioreactor itself was composed of a 9 cm long glass tube (6.5 mm O.D., 3.5 mm I.D.) in which 15 mg of the enzymatic preparation were packed between two glass wool layers. Substrate feeding was done by passing dried nitrogen, as carrier gas, through the substrate flasks. Substrates were continuously flown through the bioreactor, reacting with the lipase. The gas leaving the bioreactor was injected into a gas chromatograph for analysis. Acquisition and control of parameters (volumetric flows, molar flows, substrate and water thermodynamic activities, partial pressures, temperatures) were monitored on-line using an IBM personal computer.

A typical experiment was run at 70°C, with 15 mg of adsorbed enzyme and the total flow passing through the reactor was set at 1,000 µmol.min^{-1} giving a volumetric flow of 28.5 mL.min^{-1}. In these conditions, less than 5% of the substrates were converted allowing thus to assume that initial rates (expressed in µmoles of product formed per minute and per mg of enzyme) were measured and that inhibition by the products did not occur. Mass balances were checked at the end of each experiment; the error never exceeded 10%.

Experimental setup for organic phase synthesis

Transesterification reactions run in organic media were carried out using 50 mg of adsorbed enzyme and 4 ml of a solution containing methyl propionate, propanol and 2-methyl-2-butanol at specified concentrations. All components were previously dried over molecular sieves overnight. The concentrations for each component were calculated from molar fractions obtained using the UNIFAC group method (Hansen, 1991) and they corresponded to specified thermodynamic activities. Such calculations were performed for each experimental point of the curves displayed. The mixture was incubated at 70°C for 20 min. under magnetic stirring. Samples were taken at intervals. After a 9-fold dilution in a mixture of 2-methyl-2-butanol and methyl butyrate, the biocatalyst was removed by centrifugation and the samples were analyzed by gas chromatography.

Chromatographic assays

For the solid/gas system analyses, 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 fast ion detector (FID) for detection of all products. The column used was a CP sil 19-CB fused silica capillary column (25 m x 0.32 mm i.d. x 1.2 µm film thickness,

Chrompack, France). The split ratio was 150:1. The injector and the detector were kept at 220°C and 250°C respectively. The column temperature was held at 60°C for 1 min., then programmed to increase at 5°C.min^{-1} to 100°C. Carrier gas was nitrogen and the flow rate in the column was 2 mL.min^{-1}. Hydrogen and air were supplied to the FID at 39 and 375 mL.min^{-1} respectively. Quantitative data were obtained after integration on a HP 3396A integrator. An external standard method was used for the calculations.

For the analyses after reactions run in 2-methyl-2-butanol,a gas chromatograph with an automatic sampler (Hewlett Packard model 5890 A) equipped with a FID was used. The column was a OV 1701 (30 m x 0.32 mm i.d. x 1 µm film thickness, Chrompack, France). The split ratio was 220:1. The injector and the detector were kept at 220°C and 250°C respectively. The initial temperature was 35°C and it was increased to 80°C using two different rates : 0.5 °C.min^{-1} from 35°C to 39°C and 5°C.min^{-1} from 39°C to 80°C. Carrier gas was hydrogen and the flow rate in the column was 0.9 mL.min^{-1}. Hydrogen and air were supplied to the FID at 24 and 364 mL.min^{-1} respectively. Quantitative data were obtained after integration on a HP 3396A integrator. Methyl butyrate was used as internal standard.

RESULTS

Stability of the enzyme in the solid-gas reactor

The adsorbed enzyme was first tested for stability at 70°C. The adsorbed lipase (15 mg) was placed in the bioreactor. The total flow passing through the reactor was set at 1,000 µmol.min^{-1}. Substrates (methylpropionate and 1-propanol) thermodynamic activities (a_{MP} and a_P respectively) were maintained at 0.15 while water activity (a_W) was varied from 0 to 0.5. The products (methanol and propylpropionate) formed were quantified every 10 min. over 24 h. The results obtained showed there was a slight decrease of the enzymatic activity over 24 h.

especially for the highest water activities. However, for a_W under 0.2, more than 93 % activity was retained after 24 h (residual activities of 97.7 %, 95.2 % and 93.8 % for a_w of 0, 0.1 and 0.2 respectively). For the highest a_W, the lipase showed a lower stability with 82.3 % and 74.1 % residual activity after 24 h for a_w of 0.4 and 0.5 respectively. These results confirm the key role of water during thermodenaturation phenomena of enzymes. They were taken into account for further experiments: experiment lengths were set so that activity losses never exceeded 5 %. Moreover, most experiments were run twice and bidirectionally which means that the thermodynamic activity of the studied parameter increased in a first set of experiments and afterwards decreased so that stability and reproducibility were checked .

Kinetics and mechanism of the reaction in the solid-gas reaction

A first set of experiments was run to investigate the effect of thermodynamic activities of both substrates (methylpropionate and 1-propanol) on the initial velocity values in order to elucidate the kinetics and mechanism of the lipase. The reaction was carried out at 70°C and the thermodynamic activity of water was set near to zero because it corresponded to an optimum catalytic activity (see further results).

The results showed that the enzyme was inhibited by 1-propanol up to an optimum since bell-shaped curves were obtained when a_P was increased and a_{MP} was kept constant (Fig. 1a). The optimal a_P value is not the same depending on a_{MP}; the higher the a_{MP} value, the higher the optimal a_P. In contrast, initial velocity values increased with methylpropionate activity in the range tested (0 to 0.35) (Fig. 1b).

Reciprocal initial rates versus reciprocal a_{MP} gave different profiles depending on the a_P values (Fig. 2). For low a_P values (from 0.007 to 0.016), the plotted profiles were parallel with a decrease of the 1/V axis intercept. For higher a_P values, the plots converged to a limit of 1/Vmax on the y-axis corresponding to $1/a_{MP}$ equal to 1. Indeed, in the present case, as

thermodynamic activities are used instead of classical concentrations, the 1/Vmax axis is not the usual y-axis (for $1/a_{MP}$ values equal to zero) but a parallel axis corresponding to $1/a_{MP}$ values of 1, corresponding to the highest possible value of a_{MP}. These results agree with the Ping Pong Bi Bi mechanism with dead-end inhibition by the alcohol [29].

In this reaction sequence, the lipase reacts with methylpropionate to yield the lipase-methylpropionate complex or with propanol to yield a dead-end enzyme-propanol complex. Then the lipase-methylpropionate 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, which finally yields propylpropionate and free lipase.

The dissociation constant of the propanol-enzyme complex, K_I, can be deduced from the plot of the slopes of the converging lines from Figure 2 (corresponding to high a_P values for which an inhibitory effect is observed) versus a_P [29]. The straight line obtained (Fig. 3) gives the value of K_I at the intercept with the abscissa axis. K_I value so determined is equal to 0.019.

The rate equation for the mechanism, considering that the experiments were performed under conditions in which the influence of the products can be neglected, is given by:

$$\frac{Vi}{V_{max}} = \frac{a_P \,.\, a_{MP}}{K_{(MP)}\ \ a_P \,.\, (1 + a_P/K_I) + K_{(P)}\ .a_{MP} + a_P.a_{MP}} \qquad (1)$$

Where Vi is the initial reaction rate, Vmax is the maximum velocity or limiting rate, a_{MP} and a_P are the initial methylpropionate and propanol activities respectively, $K_{(MP)}$ and $K_{(P)}$ are the dissociation constants of the enzyme-methylpropionate (E-MP) and acyl-enzyme-propanol (Acyl-E-P) complex respectively.

According to the reaction sequence previously described, the rate constants of the reaction k_1, k_{-1}, k_2, k'_1, k'_{-1} and k'_2 are defined by :

$$\text{Enzyme} + \text{MP} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{E - MP} \xrightarrow[\text{M}]{k_2} \text{Acyl - E},$$

$$\text{Acyl - E} + \text{P} \underset{k'_{-1}}{\overset{k'_1}{\rightleftharpoons}} \text{Acyl - E - P} \xrightarrow{k'_2} \text{E} + \text{PP},$$

with $K_{(MP)} = \frac{k_{-1} + k_2}{k_1}$, $K_{(P)} = \frac{k'_{-1} + k'_2}{k'_1}$, M = methanol and PP = propylpropionate.

Equation (1) can be rearranged to:

$$Vi = \frac{V_{max} \cdot a_{MP} \cdot a_P / (K_{(P)} + a_P)}{\frac{K_{(MP)} \cdot a_P}{K_{(P)} + a_P} \cdot (1 + a_P/K_I) + a_{MP}} \qquad (2)$$

At a given activity of propanol, equation 2 can be treated as an analog of the Michaelis-Menten equation, with the apparent $V_{max(app)}$ and $K_{M(app)}$ being equal to :

$$V_{max(app)} = \frac{a_P \cdot V_{max}}{K_{(P)} + a_P} \qquad (3)$$

and

$$K_{M(app)} = \frac{a_P \cdot K_{(MP)} \cdot (1 + a_P/K_I)}{K_{(P)} + a_P} \qquad (4)$$

Thus, an alternative method for calculating the values of $K_{(MP)}$ and $K_{(P)}$ is to use a "Michaelis-Menten relationship" which is restricted by the values of $V_{max(app)}$ and $K_{M(app)}$ to a range of thermodynamic activities of propanol.

For low a_P values, the double reciprocal plot of the initial rates against the methylpropionate activities can generate $V_{max(app)}$ values : the intercept of the parallel lines with the $1/a_{MP}=1$ line

gives the $V_{max(app)}$ value for each corresponding a_P. $K_{M(app)}$ values are obtained using the same plot at the intercept of all lines (for each a_P value) with the x-axis.

Equations (3) and (4) can be rearranged to:

$$V_{max(app)} = - [K_{(P)} \cdot V_{max(app)} / a_P] + V_{max} \quad (3')$$

and

$$K_{M(app)} = K_{(MP)} \cdot \frac{a_P . (1+a_P/K_I)}{K_{(P)} + a_P} \quad (4')$$

The $V_{max(app)}$ and $K_{M(app)}$ values obtained for each a_P value were then plotted against $V_{max(app)}/a_P$ and $a_P.(1+a_P/K_I)/(K_{(P)}+a_P)$ respectively (Fig.4a and 4b). The values for V_{max}, $K_{(MP)}$ and $K_{(P)}$ so obtained were 0.620 µmol.min^{-1}.mg^{-1}, 0.047 and 0.022 respectively.

The $K_{(MP)}$ value is 2.5 times higher than the K_I value which means that propanol is a strong inhibitor. This can be confirmed by the fact that the optimum a_P values (up to which the inhibitory effect is effective) are very small (see Fig.1a) : less than 0.08 in the range of a_{MP} studied (0 to 0.35).

Determination of the kinetic parameters of the reaction in an organic medium

The transesterification reaction between methylpropionate and n-propanol was run in 2-methyl-2-butanol in order to determine the kinetic parameters. Substrate levels used were expressed in thermodynamic activities so that kinetic parameters could be compared with the ones obtained in the solid-gas reactor.

As expected, results showed that the initial velocity continuously increased with the methylpropionate thermodynamic activity, while bell-shaped curves were obtained when the thermodynamic activity of propanol was increased (for a constant methylpropionate thermodynamic activity) (results not shown). As for experiments run in the solid-gas bioreactor, double reciprocal plots were drawn (Fig. 5). Parallel lines and converging lines on the $1/a_{MP} = 1$ axis were obtained for a_P values under 0.02 and over 0.05 respectively.

These results confirm that the LBCA immobilised onto Chromosorb P AW DMCS also obeyed a Ping Pong Bi Bi mechanism when run in 2-methyl-2-butanol. The same procedure as the one described earlier was followed to determine the kinetic constants. The values for V_{max}, $K_{(MP)}$, $K_{(P)}$ and K_I so obtained were 0.316 µmol.min^{-1}.mg^{-1}, 0.651, 0.020 and 0.160 respectively.

Influence of water activity on catalytic rate in the solid-gas reactor

In this 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 water. Methylpropionate and n-propanol thermodynamic activities were kept constant (0.15) while a_W was scanned from 0 to 0.5.

On Figure 6 is reported the effect of a_W on the catalytic rate. Whatever the a_W, the initial velocity decreases when a_W increases. The plot can be separated into two parts. For a_W values under 0.1, the slope is rather slight with an activity loss of 10% for each a_W increment of 0.1. For higher a_W, the inhibitory effect of water is more important with a 17% loss for a 0.1 increase of a_W. This inhibition could be the fact of a competition between water and n-propanol for reaction with the acyl-enzyme intermediate, giving propylpropionate or propionic acid respectively. However, propionic acid was not detected in the conditions used even for the highest a_W value (a_w=0.5); this would indicate that no hydrolysis of methylpropionate occurs, unless an esterification step of propionic acid with n-propanol takes place, removing the acid formed [24].

When double reciprocal plots (1/V versus 1/a_{MP}) were drawn (Fig. 7a), converging lines near to the (1/a_{MP}=1) axis were obtained. Apparent dissociation constant, $K_{M(app)}$, calculated for each a_W value from Fig. 7a was then plotted versus a_W for the determination of the dissociation constant of the water-enzyme complex, K'_I. This plot gave a line for a_W superior

to 0.1 (Fig. 7b). The water inhibition constant so determined is equal to 0.128 which is more than five times higher than the inhibition constant for propanol.

Strange as it may seem to consider water as a competitive inhibitor, as n-propanol is, this may be exactly what it is doing. For the lowest a_W, water probably exerts a role in making the enzyme structure more flexible and mobile, increasing the turnover rate. This activating effect is opposed to the competitive inhibition effect therefore the initial rate decreases slowly. But as long as the enzyme hydration level is sufficient for optimal catalytic activity, competitive inhibition by water becomes predominant. A water activity around 0.1 for optimal catalytic activity seems reasonable when making reference to other study from Chamouleau et al. [30] who observed a decrease of reaction rates with water activity for a_W higher than 0.07 when using CALB for the esterification of fructose and palmitic acid. Although the immobilization support used in this work was polypropylene, the results obtained are comparable to those of the present study. Indeed, it was shown that the shape of the rate/a_W profile is mainly an intrinsic property of the enzyme molecules used and is therefore not significantly affected by a change of support or solvent [31].

DISCUSSION

The kinetic results for the alcoholysis of methylpropionate with n-propanol in a continuous solid/gas bioreactor showed that the initial reaction rates fitted well a Ping Pong Bi Bi mechanism with competitive inhibition by the alcohol. Similar results have been reported either in organic media or in supercritical carbon dioxide (see the introduction) but this is the first work run in the gas phase for which a mathematical model can be fitted to the experimental results. The constants obtained in the present study are expressed in term of thermodynamic activities and are therefore difficult to compare with values expressed in concentrations reported in the literature. Therefore kinetic constants were determined in 2-

methyl-2-butanol using the UNIFAC group contribution method [23]. The values obtained are 0.651 and 0.160 for the ester dissociation constant and the propanol inhibition constant respectively. Both of these constants are far higher than the ones obtained when the reaction is performed in the solid-gas bioreactor (0.047 and 0.019 respectively). Moreover the maximal velocity V_{max} calculated in the organic medium (0.316 µmol.min^{-1}. mg^{-1}) is half the one obtained in the solid-gas reactor (0.620 µmol.min^{-1}. mg^{-1}) . These results are in accordance with lower diffusional limitations in gas than in liquid media. Similar conclusion was obtained in studies comparing kinetic parameters for ester synthesis by lipase-catalyzed transesterification in supercritical carbon dioxyde ($SCCO_2$) and in hexane [10]. Lower dissociation constants in $SCCO_2$ than in hexane were obtained and the lower diffusional limitations in $SCCO_2$ was put forward.

The present study also revealed that water, which is neither a substrate nor a product of the reaction, acted as a competitive inhibitor for the enzyme. The mechanism which can be proposed is summarized on scheme 1.

In a previous work run in hexane for the esterification of dodecanol and decanoic acid, Valivety et al. [3] noticed that an increase in water activity led to an increase in the dissociation constant of the enzyme for the acyl substrate; they therefore concluded that water acted as a competitive inhibitor. The authors stated that water bound close to the active site may be in position to obstruct binding of the acyl-substrate. As reported by Uppenberg et al. [28], the region around the catalytic serine (Ser-105) is polar in nature so the hydroxyl of the alcohol or of water may bind to this region to form the dead-end complex. The hydrophobic part of the alcohol could bind either to the ester acyl part binding site or to the ester alkyl part one [8]. This could account for the lower affinity of water (K_I=0.128) than n-propanol (K_I=0.019) for the enzyme. Indeed, in the case of water, the binding to the enzyme would not be stabilized.

These results emphasize the great advantage of the solid/gas system presented here over all the systems described to date; indeed, some literature reports on the effect of water level on the acyl substrate dissociation constant values, showing that water is acting to consistently increase the K_M values [32, 33]. But as concentrations were used rather than thermodynamic activities, the changes in substrate concentration may have altered enzyme hydration by modifying the capacity of the organic phase to dissolve water in competition with the enzyme [34]. Therefore no comment was made on possible mechanism even if the general trends observed were probably correct. Other studies are made after pre-equilibration of substrates, solvent and enzyme at a known a_W [35, 36] but these studies are rigorous only if initial rates are measured in particular when water is a product of the reaction (for esterification reactions for example) or when synthesis is long.

Experimental studies are currently in progress to determine the effect of the alcohol polarity on its inhibitory character in order to precise the hypothesis on the mechanism of the reaction.

Aknowledgements

We want to thank the ANVAR Poitou Charentes for their financial support, within the framework of technological transfer help.

REFERENCES

[1] Chulalaksananukul, W., Condoret, J.S., Delorme, P. and Willemot, R.M. (1990) FEBS Lett. 276 : 181-184.

[2] Chulalaksananukul, S., Longo, M.A., Chulalaksananukul, W., Condoret, J.-S. and Combes, D. (1999) AFINIDAD LVI, 480: 121-125.

[3] Valivety, R.H., Halling, P.J. and Macrae, A.R. (1993) Biotechnol. Lett. 15: 1133-1138.

[4] Lai, D.T., Hattori, N. and O'Connor, C.J. (1999) JAOCS 76: 845-851.

[5] Rizzi, M., Stylos, P., Riek, A. and Reuss, M. (1992) Enzyme Microb. Technol. 14: 709-714.

[6] Straathof, A.J.J., Rakels, J.L.L. and Heijnen, J.J. (1992) In: Biocatalysis in non-conventional media (Tramper, J., Vermuë, M.H., Beeftink, H.H. and von Stockar, U., Eds.). Elsevier, Amsterdam, 137-144.

[7] Valivety, R.H., Halling, P.J., Peilow, A.D. and Macrae, A.R. (1992) Biochim. Biophys. Acta 1122: 143-146.

[8] Martinelle, M. and Hult, K. (1995) Biochim. Biophys. Acta. 1251: 191-197.

[9] Janssen, A.E.M., Sjursnes, B.J., Vakurov, A.V. and Halling, P.J. (1999) Enzyme Microb. Technol. 24: 463-470.

[10] Chulalaksananukul, W., Condoret, J.S. and Combes, D. (1993) Enzyme Microb. Technol. 15: 691-698.

[11] Goddard, R., Bosley, J. and Al-Duri, B. (2000a) J. Chem. Technol. Biotechnol. 75: 715-721.

[12] Goddard, R., Bosley, J. and Al-Duri, B. (2000b) J. of Supercritical Fluids 18: 121-130.

[13] Lamare, S. and Legoy, M.6D. (1997) Biotechnol. Bioeng., 56: 1-8.

[14] Serralha, F.N., Lopes, J.M., Lemos, F., Prazeres, D.M.F., Aires-Barros, M.R., Cabral, J.M.S. and Ribeiro, F.R. (2001) J. Mol. Catal. B 11: 713-718.

[15] Bell, R.P., Critchlow, J.E. and Page, M.I. (1974) J. Chem. Soc., Perkin Trans. II. 66-70.

[16] Van Tol., J.B.A., Odenthal, J.B., Jongejan, J.A. and Duine J.A. (1992) In: Biocatalysis in non-conventional media (Tramper, J., Vermuë, M.H., Beeftink, H.H. and von Stockar, U., Eds.). Elsevier, Amsterdam, 229-235.

[17] Dordick, J.S. (1992) Biotechnol. Prog. 8: 259-267.

[18] Halling, P.J. (1994) Enzyme Microb. Technol. 16: 178-206.

[19] Kraut, J. (1988) Science 242: 533-540.

[20] Janssen, A.E.M. and Halling, P.J. (1994) J. Am. Chem. Soc. 116: 9827-9830.

[21] Janssen, A.E.M., Vaidya, A.M. and Halling, P.J. (1996) Enzyme Microb. Technol. 18: 340-346.

[22] Reimann, A., Robb, D.A. and Halling, P.J. (1994) Biotechnol. Bioeng. 43: 1081-1083.

[23] Hansen, H.K., Rasmussen, P., Fredenslund, A., Schiller, M. and Gmehling, J. (1991) Ind. Eng. Chem. Res. 30: 2355-2358.

[24] Lamare, S. and Legoy, M.D. (1995) Biotechnol. Bioeng., 45: 387-397.

[25] Wounderberg-Van Oosterom, M, Van Rantwijk, F., Sheldon, R.A. (1996) Biotechnol. Bioeng. 49: 328-333.

[26] Bousquet, M.-P., Willemot, R.-M., Monsan, P. and Boures, E. (1999) Biotechnol. Bioeng. 62: 226-234.

[27] Uppenberg J., Hansen M.T., Patkar S. and Jones T.A. (1994) Structure. 4 : 293-308.

[28] Uppenberg J., Öhrner N., Norin M., Hult K., Kleywegt G.J., Patkar S., Waagen V., Anthonsen T. and Jones T.A. (1995) Biochemistry. 34 : 16838-16851.

[29] Segel I.H. (1975) John Wiley and Sons, New York.

[30] Chamouleau, F., Coulon, D., Girardin, M. and Ghoul, M. (2001) J. Mol. Catal. B. 11: 949-954.

[31] Valivety, R.H., Halling, P.J., Peilow, A.D. and Macrae, A.R. (1994) Eur. J. Biochem. 222: 461-466.

[32] Reslow M., Adlercreutz P. and Mattiasson B. (1987) Appl.Microbiol.Biotechnol. 26 : 1-6.

[33] Marty A., Chulalaksananukul W., Willemot R.M. and Condoret J.S.(1992) Biotechnol.Bioeng. 39 : 273-280.

[34] Wehtje E. and Adlercreutz P. (1997) Biotechnol. Bioeng. 55 : 798-806.

[35] Svensson I., Wehtje E., Adlercreutz P. and Mattiasson B. (1994) Biotechnol.Bioeng. 44 : 549-556.

[36] Dudal Y. and Lortie (1995) Biotechnol.Bioeng. 45 : 129-134.

DIAGRAM LEGENDS

Figure 1: Effect of substrate thermodynamic activities on initial rates.

Synthesis was carried out in the solid/gas bioreactor at 70°C, at a molar flow of 1,000 µmol.min^{-1} in the presence of 15 mg of adsorbed CALB onto Chromosorb AW DMCS. Methylpropionate and propanol activities were varied from 0 to 0.35. Water activity was set near to 0.

Figure 2: Reciprocal initial rates versus reciprocal a_{MP} for different a_P values.

Synthesis was carried out in the solid/gas bioreactor at 70°C, at a molar flow of 1,000 µmol.min^{-1} in the presence of 15 mg of adsorbed CALB onto Chromosorb AW DMCS. Water activity was set near to 0.

Figure 3: Determination of the dissociation constant of the enzyme-propanol complex (K_I) in the solid/gas bioreactor

Figure 4: Determination of V_{max}, $K_{(P)}$ and $K_{(MP)}$ in the solid/gas bioreactor

Figure 5: Reciprocal initial rates versus reciprocal a_{MP} for different a_P values.

Synthesis was carried out at 70°C, in the presence of 50 mg of adsorbed CALB onto Chromosorb AW DMCS and 4 mL of a solution containing methyl propionate, propanol and 2-methyl 2-butanol at concentrations corresponding to the displayed thermodynamic activity values for both substrates.

Figure 6: Effect of water thermodynamic activity on initial rates.

Synthesis was carried out at 70°C, at a molar flow of 1,000 µmol.min^{-1} in the presence of 15 mg of adsorbed CALB onto Chromosorb AW DMCS. Methylpropionate and propanol thermodynamic activities were fixed at 0.15 and a_W was varied from 0 to 0.5.

Figure 7: Reciprocal initial rates versus reciprocal a_{MP} for different a_W values (Fig. 6a) - Determination of the dissociation constant of the enzyme-water complex (K'_I) (Fig. 6b)

Synthesis was carried out at 70°C, at a molar flow of 1,000 µmol.min^{-1} in the presence of 15 mg of adsorbed CALB onto Chromosorb AW DMCS. Propanol thermodynamic activity was fixed at 0.15 and a_W was varied from 0 to 0.5.

Figure 1

Fig. 1a

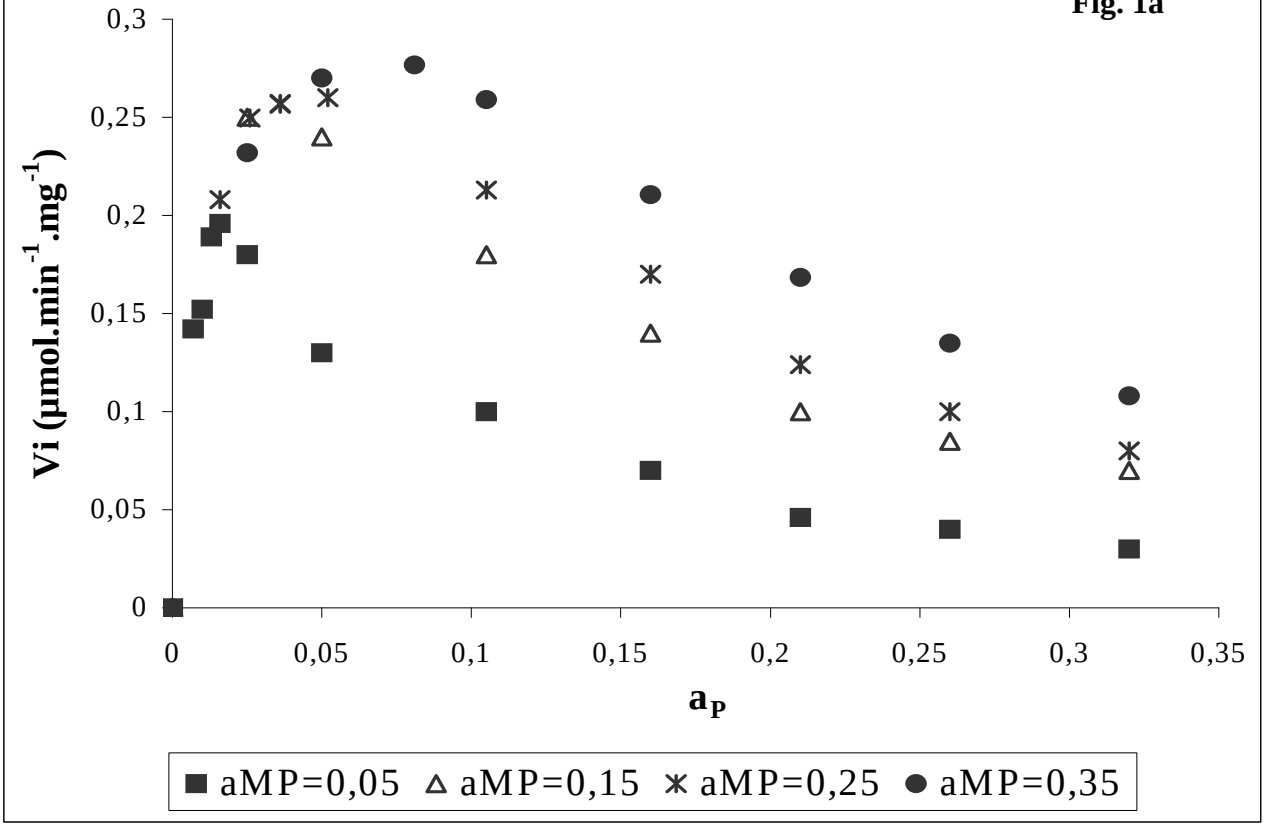

Fig. 1b

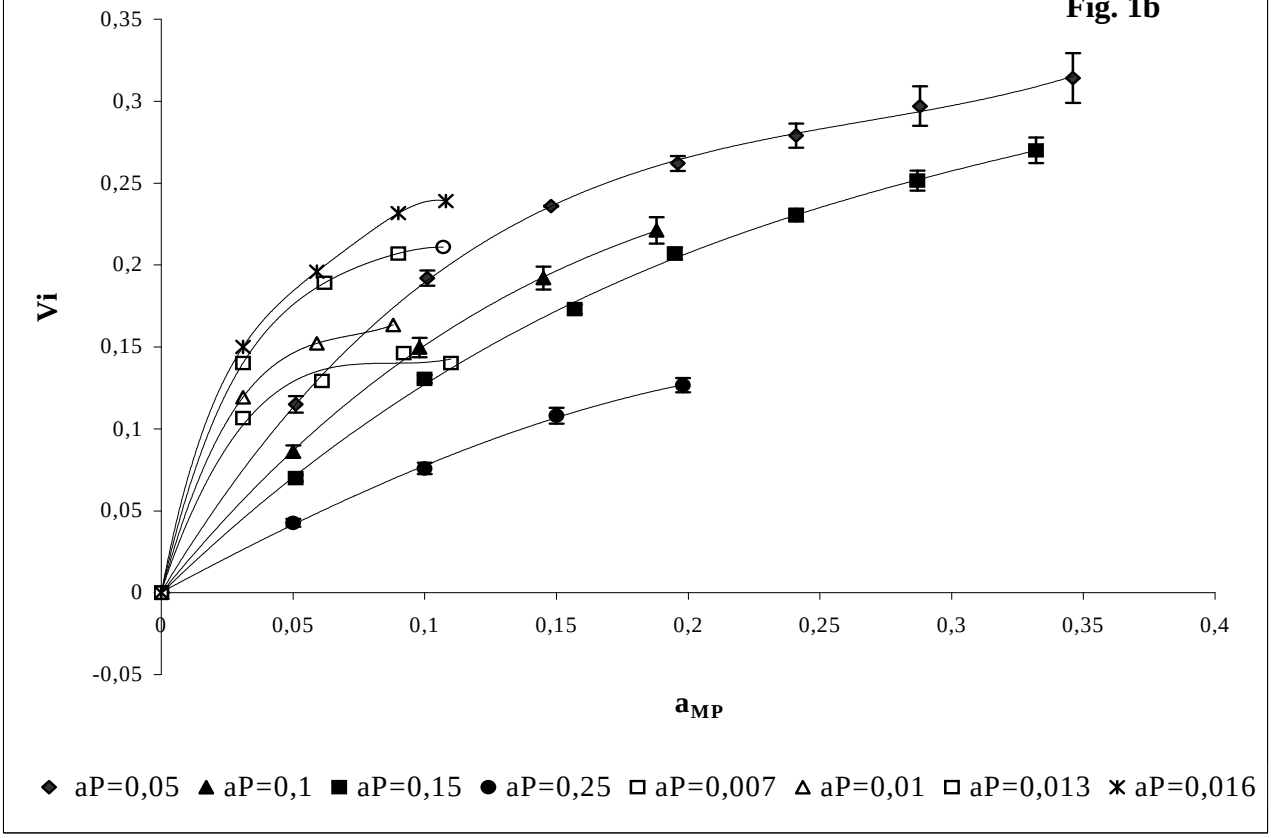

FIGURE 2

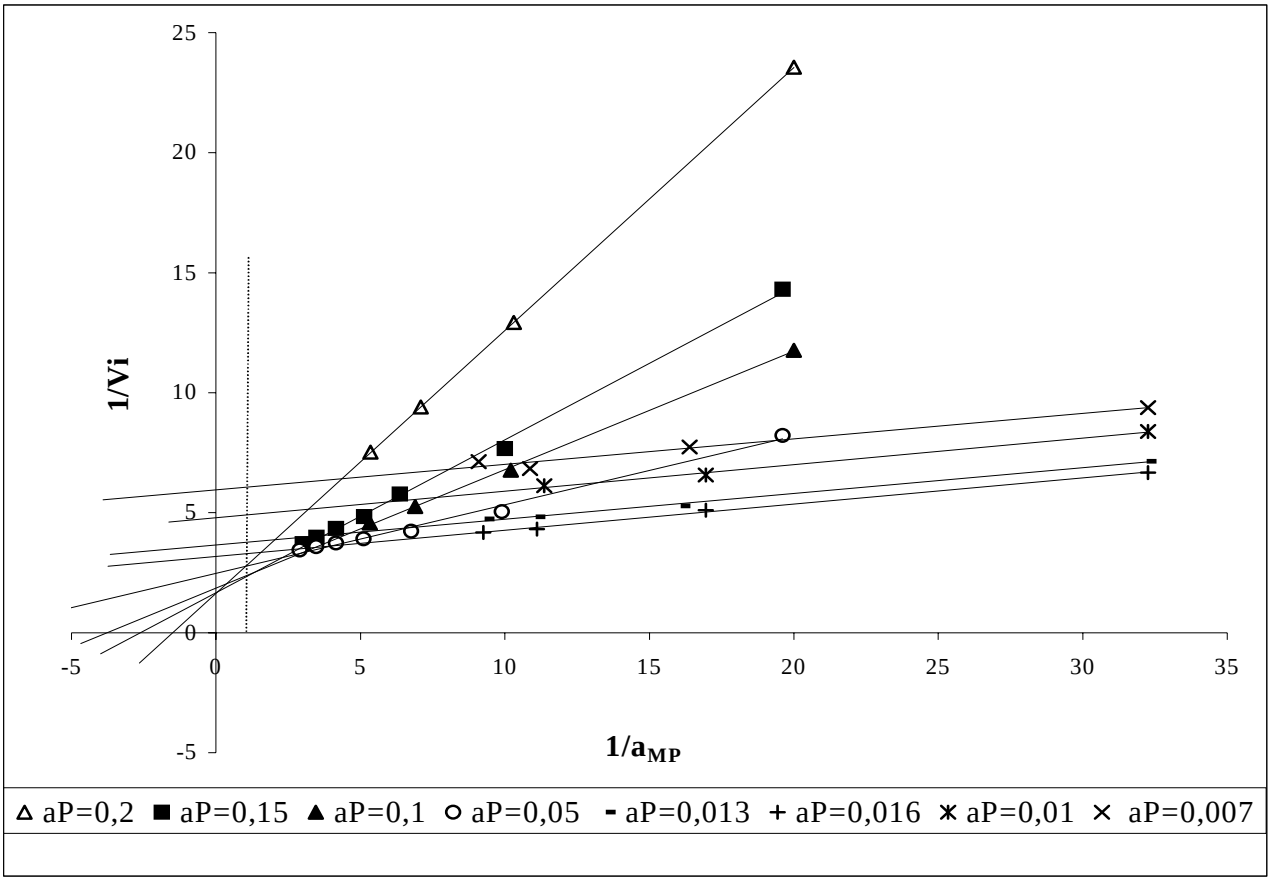
25
20
15
10
5
0
-5
1/Vi
-5
0
5
10
15
20
25
30
35
1/a_MP
aP=0,2
aP=0,15
aP=0,1
aP=0,05
aP=0,013
aP=0,016
aP=0,01
aP=0,007

FIGURE 3

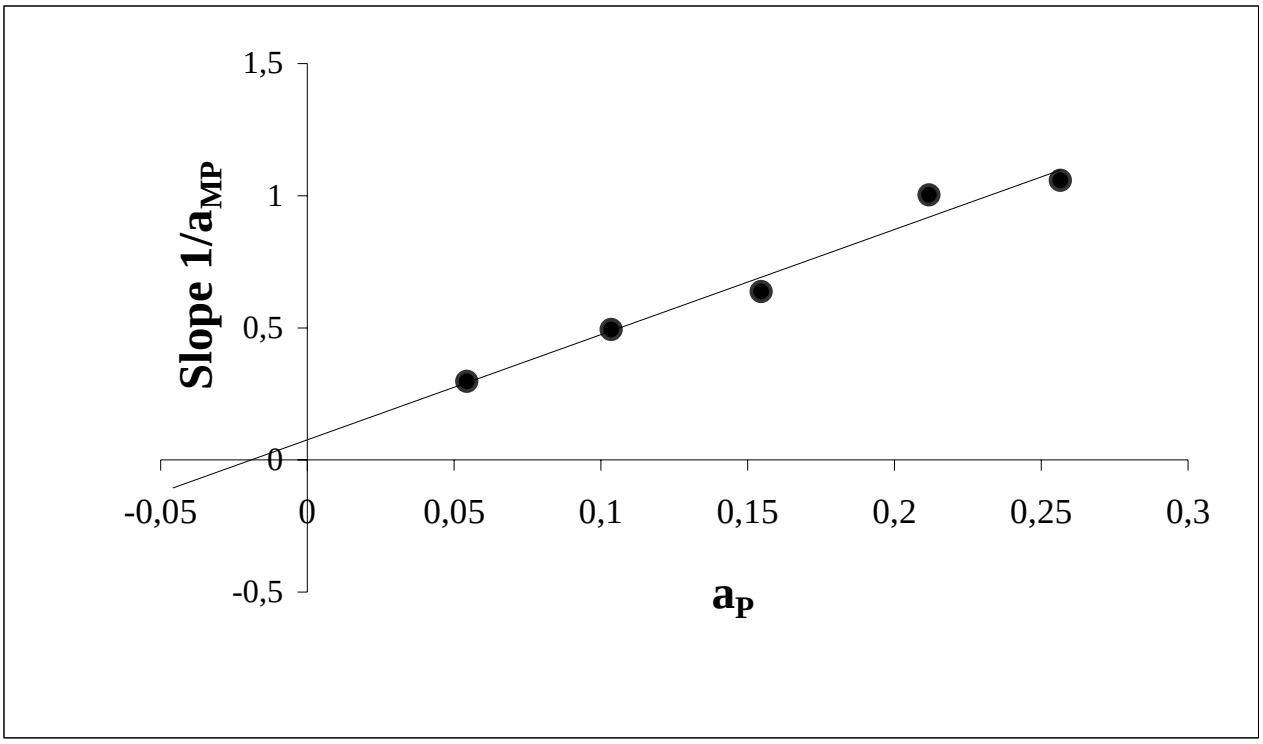

FIGURE 4

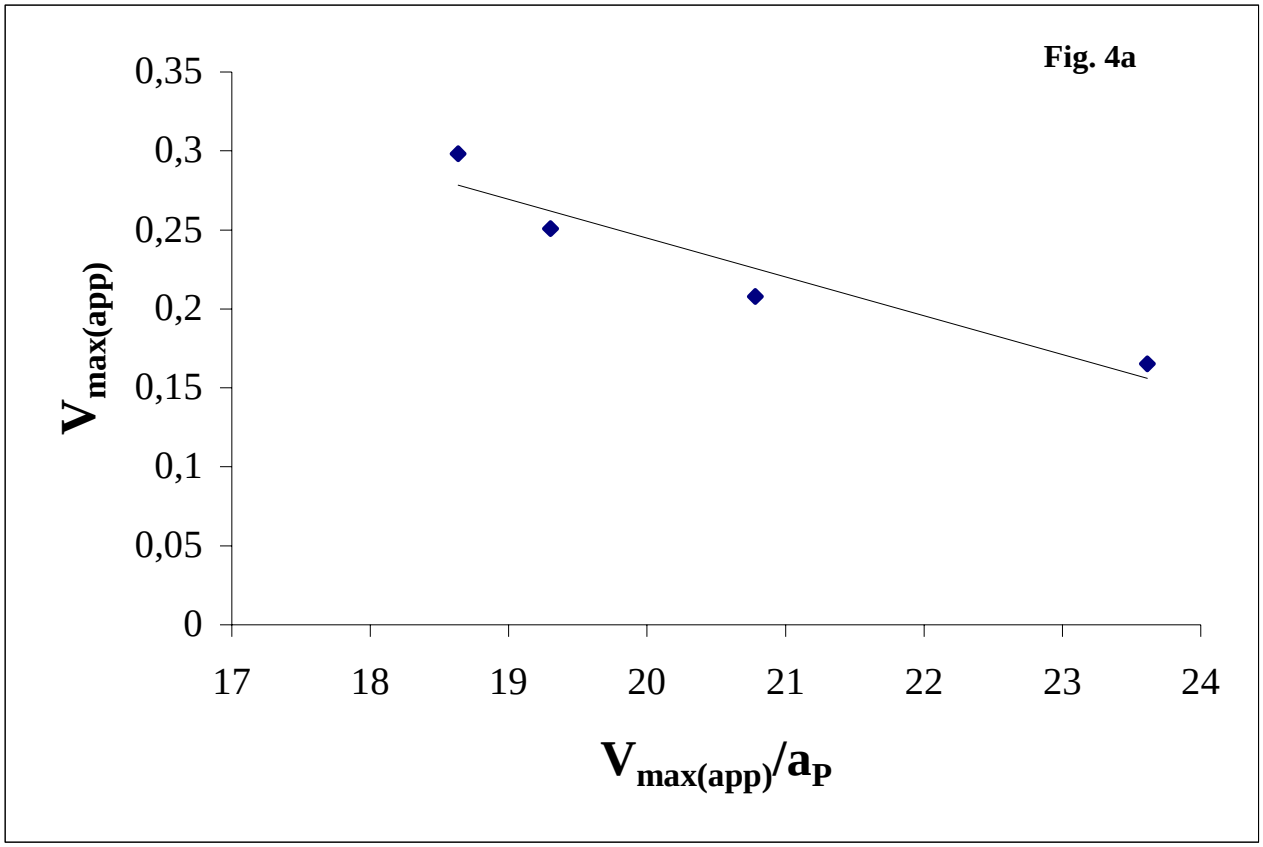
Fig. 4a
0,35
0,3
0,25
0,2
0,15
0,1
0,05
0
17
18
19
20
21
22
23
24
$V_{max(app)}$
$V_{max(app)}/a_P$

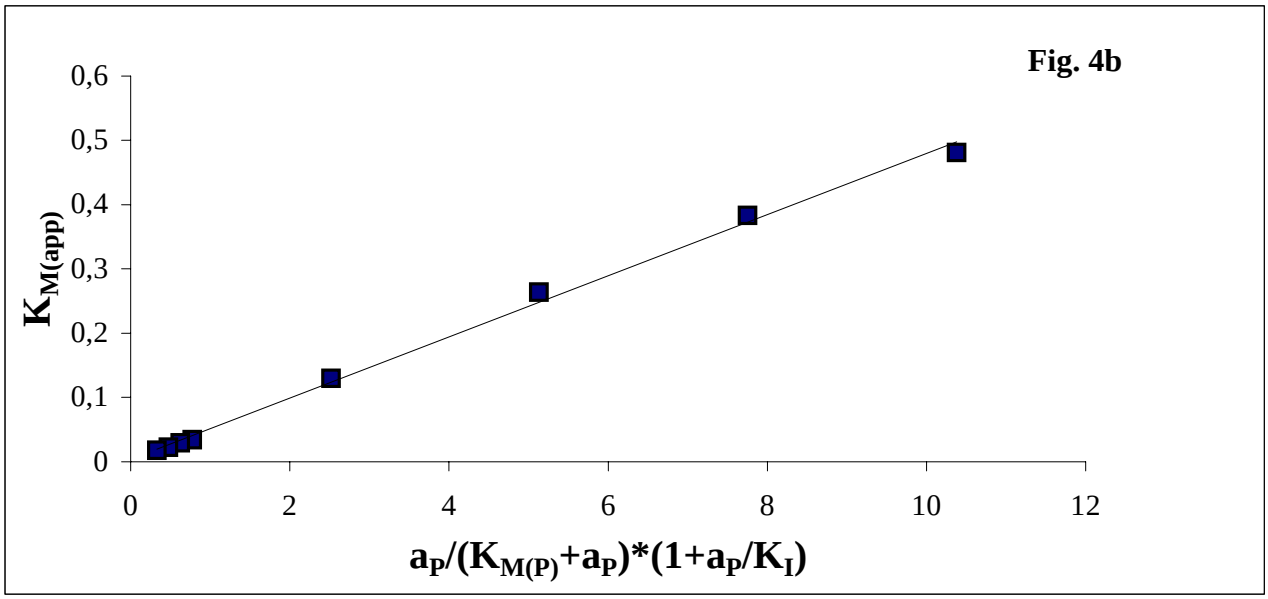
Fig. 4b
0,6
0,5
0,4
0,3
0,2
0,1
0
0
2
4
6
8
10
12
$K_{M(app)}$
$a_P/(K_{M(P)}+a_P)*(1+a_P/K_I)$

FIGURE 5

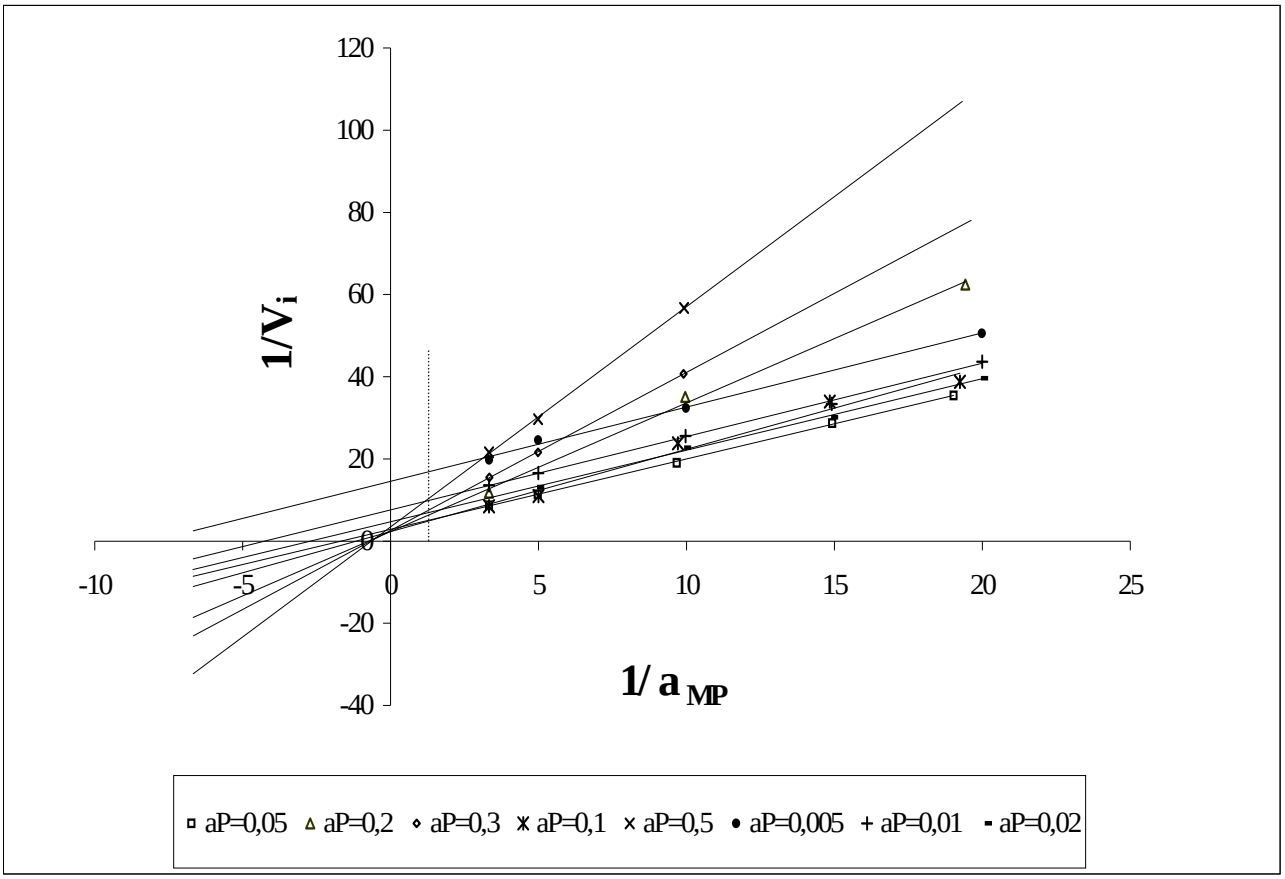
120
100
80
60
40
20
0
-20
-40
-10
-5
0
5
10
15
20
25
1/Vi
1/ a MP
aP=0,05
aP=0,2
aP=0,3
aP=0,1
aP=0,5
aP=0,005
aP=0,01
aP=0,02

FIGURE 6

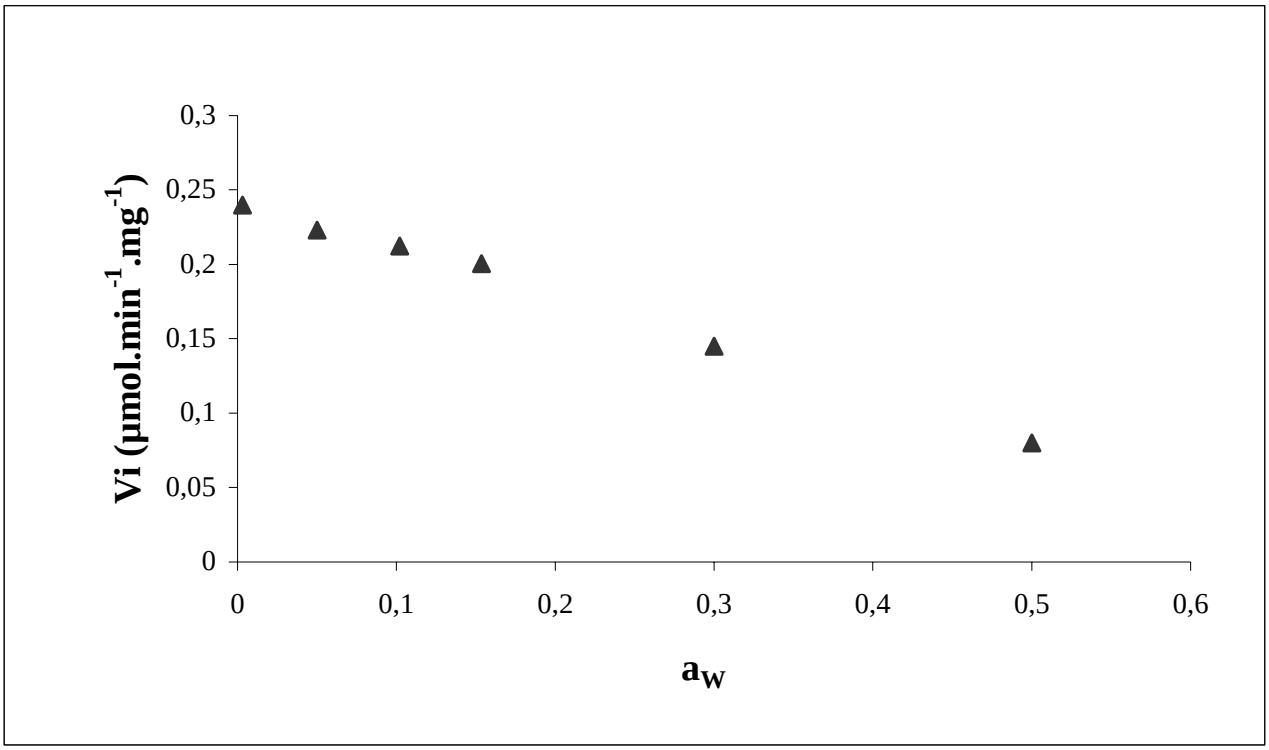

FIGURE 7

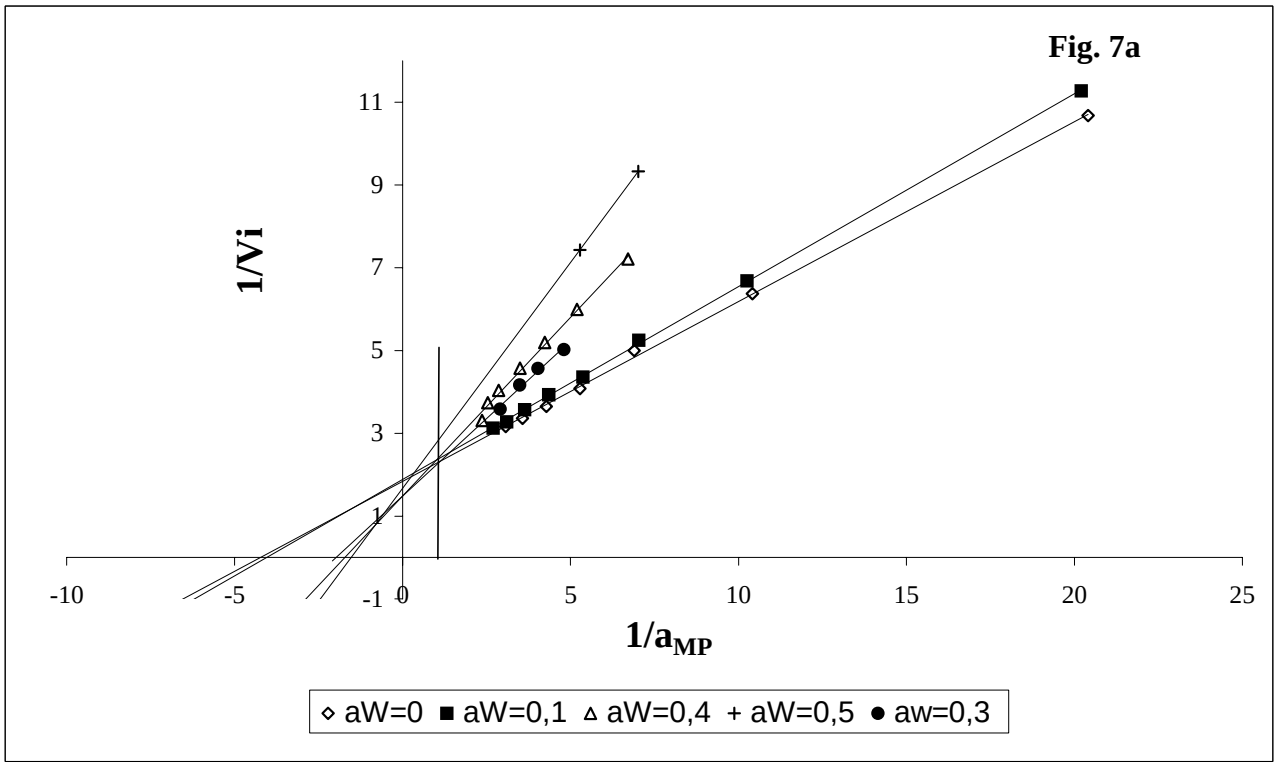
Fig. 7a
1/Vi
11
9
7
5
3
1
-1
-10
-5
0
5
10
15
20
25
1/a_MP
◇ aW=0 ■ aW=0,1 △ aW=0,4 + aW=0,5 ● aw=0,3

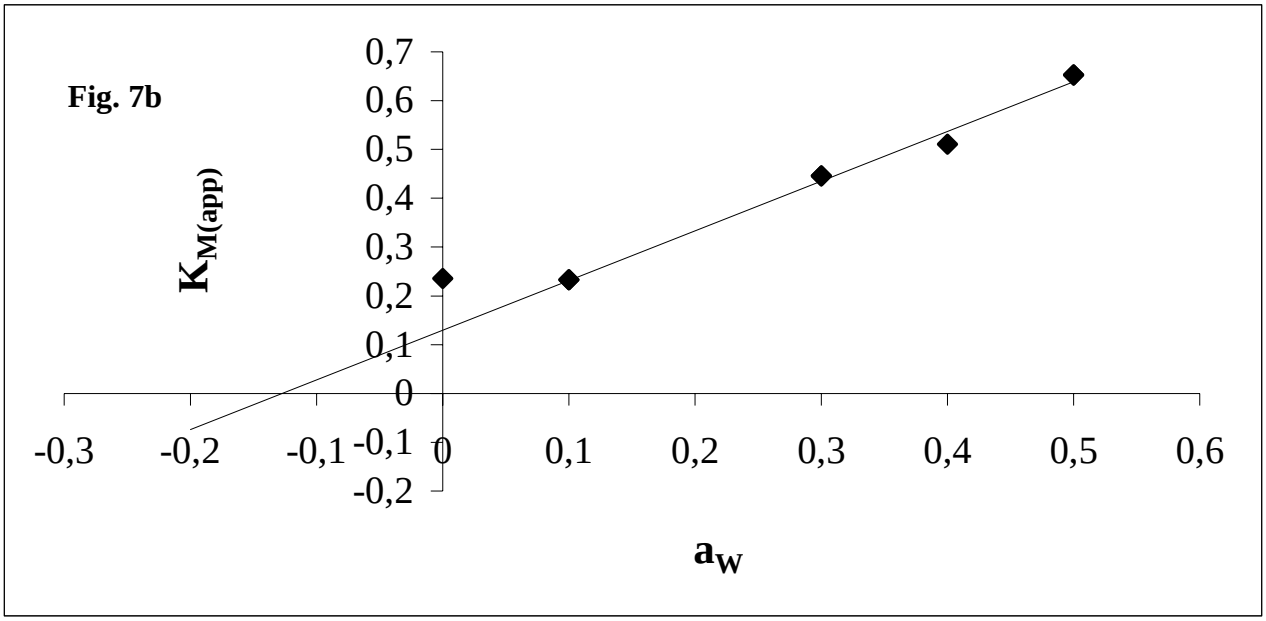
Fig. 7b
K_M(app)
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
-0,1
-0,2
-0,3
-0,2
-0,1
0
0,1
0,2
0,3
0,4
0,5
0,6
a_W

SCHEME 1