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Kinetic study of the alkyl flavonoid ester prunin 6″-*O*-laurate synthesis in acetone catalysed by immobilised *Candida antarctica* lipase B

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ABSTRACT

Alkyl prunin esters are new compounds, which are soluble in lipophilic media. They possess antioxidant and antibacterial properties, so they may have useful applications. The current work studied the kinetic of prunin 6″-*O*-laurate synthesis catalysed by Novozym 435 from vinyl laurate and prunin in acetone completely solubilised, forming colloids or scattered in solid state, according to its concentration in the reaction media.

The kinetic study was determined at 50 °C using initial concentrations between 20 and 220 mM for prunin and between 20 and 4000 mM (solvent free system) for vinyl laurate. When prunin completely solubilised or forming colloids was used, a model based on mechanism ordered Bi Bi without inhibition, neither by alcohol nor acyl group, which was the best fit in the initial rate data. The determined model was used to simulate initial reaction rate and these values were plotted against the experimental data. The model was consistent with the experimental data (slope 0.97 ± 0.01, R^2 0.993, n = 72), even on solvent-free systems.

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1. Introduction

Naringin is one of the most common citrus flavanone glycosides which is mainly found in grapefruits and sour oranges and it can be easily obtained from citrus industry waste [1]. Our laboratory developed a very efficient biocatalytic method to obtain rhamnose and prunin from supersaturated naringin solutions [2]. Rhamnose is a valuable substance in the food industry, whereas prunin is a less known and studied substance, which according to our knowledge is not commercially available. Prunin could have useful uses in the pharmaceutical and food industry due to its biological activities [3–5]. However, its application will be limited by its low solubility in lipophilic media, an issue that may be solved by the addition of a hydrophobic chain to the flavonoid.

In a previous study we showed the possibility of synthesising alkyl prunin esters (Fig. 1) from vinyl esters and prunin in different organic solvents using *Candida antarctica* lipase B (Novozym 435) and *Rhizomucor miehei* lipase (Lipozyme RM IM) as catalysts. A structural modification successfully led to an increase in solubility of the prunin esters in lipophilic media and did not affect the antiradical activity of the flavanone glucoside [6]. Among them, the prunin 6″-*O*-laurate already has proved to be an important antimicrobial agent against both bacteria and moulds [7–9]. The promising results presented by this compound drove us to study the kinetic governing its synthesis in view of the possible production on a larger scale.

There are certain studies that report on the feasibility of the synthesis of other flavonoid esters, using lipases and proteases [10,11] both in organic and non-conventional media, but few authors have assayed the kinetics. For example, Kodelia and Kolisis [12] studied the esterification of rutin with trichlorethylbutyrate in pyridine using a commercial *Bacillus subtilis* protease and Kontogianni et al. [13] examined the synthesis of naringin decanoate in *tert*-butanol catalysed by Novozym 435.

The aim of this work was to determine the mechanism and the kinetic law for the synthesis of the prunin 6″-*O*-laurate (PL), an alkyl flavonoide ester in acetone as solvent, using the commercial preparation Novozym 435.

2. Materials and methods

2.1. Materials

Molecular sieves (sodium aluminium silicate) with 4 Å pore diameter were purchased from Sigma (USA) and vinyl laurate (VL) was bought from Fluka (USA). Prunin (P) was obtained according to Soria and Ellenrieder [14] through enzymatic hydrolysis of naringin. Novozym 435 (*C. antarctica* lipase B immobilised in an acrylic resin) was a gift from Novozymes Latin America Limited

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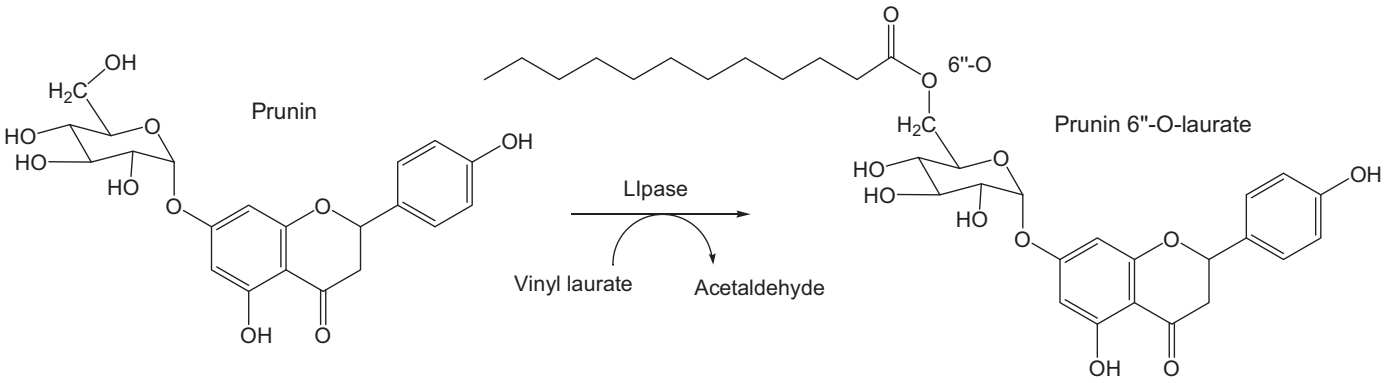

Fig. 1. Scheme of enzymatic synthesis of prunin 6″-*O*-laurate catalysed by a lipase.

(Brazil). The solvents used for analytical methods were of HPLC grade, whereas acetone was of analytical grade.

Water was removed from acetone and vinyl laurate by adding molecular sieves (30 mg mL^{-1}). The resulting mixtures were kept in the presence of the adsorbent.

2.2. Determination of initial rates

Different quantities of P (60–1200 μmol) and 200 mg of molecular sieves were placed in a screw-capped vial with a Teflon seal. Then 3 mL of an appropriate mixture of VL and acetone (VL from 10 to 4000 mM) was added and the resulting reaction media was incubated with gentle shaking at 50 °C. The reaction was started by adding 50 mg of immobilised enzyme under agitation at 200 rpm. At regular time intervals, 50 μL aliquots were extracted and appropriate dilutions of the samples were analysed by HPLC. The initial reaction rate for each initial substrate concentration was determined from the slope at time zero of the straight line that best fit product concentration versus time. The conversions reached were always less than 10%. All experiments were carried out in duplicate.

2.3. Analytical method

The concentrations of P and prunin 6″-*O*-laurate (PL) were determined by HPLC (Gilson, France) using a GraceSmart RP 18 by Grace, USA (25 cm long, 4 mm internal diameter, and 5 μm particle size). Samples (20 μL) were eluted isocratically with acetonitrile/water (80/20) at 0.8 mL min^{-1}. Elution profiles were monitored at 280 nm.

The peak areas were used directly to evaluate the conversion by dividing the area of PL peak by the area of the two peaks detected (P at 2.7 min and PL at 5.8 min). Then, the conversions were multiplied by the initial prunin concentrations to determine the PL concentrations. This calculation is based on having found experimentally that the response factor of both compounds were equal.

2.4. Solubility of prunin in acetone and acetone-vinyl laurate mixtures

Diverse P containing systems (P from 20 to 400 mM) were prepared in acetone and in acetone–VL mixtures (VL from 80 to 4000 mM) at 50 °C. The systems were centrifuged at 10,000 rpm for 5 min after incubate with gentle shaking for 20 min at 50 °C. Samples were taken from the supernatant and the P concentrations were measured by HPLC.

2.5. Data analysis

Statistical analysis and graphics were carried out using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego, CA, USA, www.graphpad.com.

3. Results and discussion

3.1. Mass transfer effect

When immobilised enzymes are used substrates must spread from the bulk of the liquid to the external particle surface and then enter the pores. Hence, the kinetics of a catalysed reaction could be controlled by limitation of external or intra-particle mass transfer.

The mass transfer limitation can be overcome by carrying out the reaction at adequate agitation rates. In this work, the effect of the agitation speed was studied in the range of 70–250 rpm (20 mM prunin, 40 mM vinyl laurate, 67 mg mL^{-1} molecular sieves, 17 mg mL^{-1} enzyme at 50 °C). Initial rate increased with increasing agitation speed until 150 rpm (0.016 ± 0.001 mM min^{-1}) and was stable at higher rates. All further experiments were carried out at 200 rpm, as it can be reasonably accepted that at this rate the external mass transfer limitation is insignificant.

Several authors have studied intra-particle mass transfer limitations for Novozym 435 and based on different arguments they have concluded that the effect can be neglected [15,16]. This could be attributed to the fact that most of enzyme in Novozym 435 is located in an external shell of the Lewatit bead with a thickness of 80–100 μm [17,18].

3.2. Enzyme concentration effect

The effect of the catalyst concentration was tested using concentrations between 3.3 and 16.7 mg mL^{-1} (20 mM prunin, 100 mM vinyl laurate, 67 mg mL^{-1} molecular sieves, 200 rpm stirring rate at 50 °C). A first order dependence was observed between the initial rate and the catalyst concentration (slope 0.98 ± 0.05 μM min^{-1} mg $enzyme^{-1}$), which is consistent with a kinetically controlled reaction.

3.3. Solubility of prunin in acetone–vinyl laurate (VL) mixtures

Solubility tests of prunin helped to differentiate, based on physical appearance, three type of systems: (a) solution: prunin completely solubilised, (b) colloid: a turbid prunin solution, (c) suspension: systems with prunin precipitated. The supernatant P concentrations in the three kinds of systems decreased with increasing VL concentration when the systems were kept at 50 °C for 20 min (Table 1).

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Table 1
Percentage of prunin still dissolved after 20 min at 50 °C, depending on the media composition. 0.1% >means lower than 0.1%. White box: solutions; pale grey box: colloids; dark grey box: suspensions.

Vinyl laurate in acetone (mM)	Initial prunin concentration (mM)				
	20	80	220	300	400
	Prunin still dissolved after 20 min at 50 °C (%)				
0 (pure acetone)	100%	99%	99%	47%	20%
80	100%	99%	69%	<0.1%	<0.1%
400	100%	87%	46%	<0.1%	<0.1%
800	100%	87%	31%	<0.1%	<0.1%
1600	100%	69%	23%	<0.1%	<0.1%
3000	99%	1.1%	0.2%	<0.1%	<0.1%
4000 (pure vinyl laurate)	55%	1.1%	0.2%	<0.1%	<0.1%

3.4. Initial rates

Initial velocities were measured with substrate concentrations between 20 and 400 mM for P and between 20 and 4000 mM for VL. Fig. 2 shows the profiles of the initial rate versus varying concentrations of one of the substrates, while the other one was kept constant.

3.5. Kinetic model

For systems with both colloidal and solubilised prunin, an increase in any of the substrate concentration produced an increase in initial rate. For the systems with prunin suspension the initial rate decreased abruptly. For this reason, to deduce the kinetic model the data was limited to those in which precipitation of P was negligible (P from 20 to 220 mM for all the VL concentrations).

Graphical analysis of both initial velocity plot and their respective inversion plot (Fig. 3), using prunin completely solubilised or colloidal, indicated the absence of inhibition.

Synthesis of prunin 6″-*O*-laurate (PL) is an enzymatic reaction between two substrates that give two products (Bi Bi reaction). Considering classical kinetics, this reaction could be classified into an ordered, random or Ping Pong mechanism. In ordered or random Bi Bi mechanisms both substrates must link to the enzyme before a product can be released, while in the Ping Pong Bi Bi mechanism one or more products must be released before all substrates have reacted. In an ordered mechanism the entry and exit of substrates to and from the active site have a determined sequence, whereas in a random mechanism the linkage or release of products is random. In the case of lipase-catalysed reactions it has been established that the first step in synthesis, involves the linkage of an acyl donor group to the enzyme forming an acyl–enzyme complex [19]. Therefore, the random mechanism was ruled out. The equations for Bi Bi systems by Segel [29] when only initial velocities and approximation of the stationary state are considered are presented below:

Ordered Bi Bi mechanism:

$$v = \frac{V_{max}[VL][P]}{K_{VL}K_{mP} + K_{mP}[VL] + K_{mVL}[P] + [VL][P]}$$

Ping Pong Bi Bi mechanism:

$$v = \frac{V_{max}[VL][P]}{K_{mP}[VL] + K_{mVL}[P] + [VL][P]}$$

where V_{max} is the maximal initial velocity, K_{VL} is the equilibrium dissociation constant for the binary enzyme–acyl donor complex (E–VL), K_{mP} and K_{mVL} are groups of rate constants which have the same significance of the Michaelis–Menten kinetic constants for each substrate.

The mechanisms for Bi Bi systems can be represented by an Michaelis–Menten equation with apparent parameters, $V = V_{max}^{ap} \times [S]/(K^{ap} + [S])$, provided that the concentration of one substrate remains constant. The observed initial rate for both completely solubilised and colloidal prunin could be adjusted by nonlinear regression using this equation. Deviations from this model were not statistically significant with P values ranging from 0.2 to 0.8.

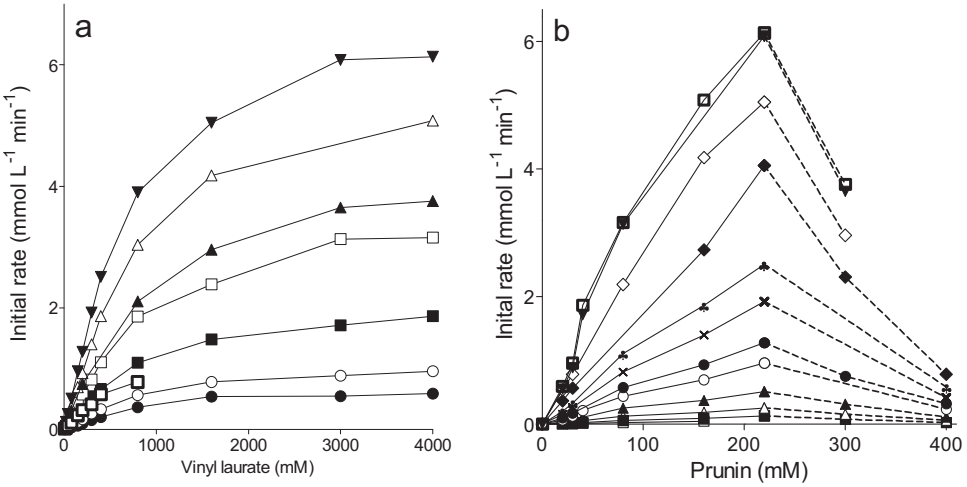

Fig. 2. (a) Initial rate versus vinyl laurate concentration (mM) at different initial concentrations of prunin (mM): ● 20, ○ 30, 40 mM, □ 80 mM, △ 160, ▼ 220, ▲ 300 mM and □ 400 mM. (b) Initial rate versus prunin concentration (mM) at different initial concentrations of vinyl laurate: □ 10, 20, △ 40, ▲ 80, ○ 150, ● 200, × 300, ♣ 400, ♦ 800, ◊ 1600, ▼ 3000 and □ 4000. Dashed lines group values obtained with prunin in suspension.

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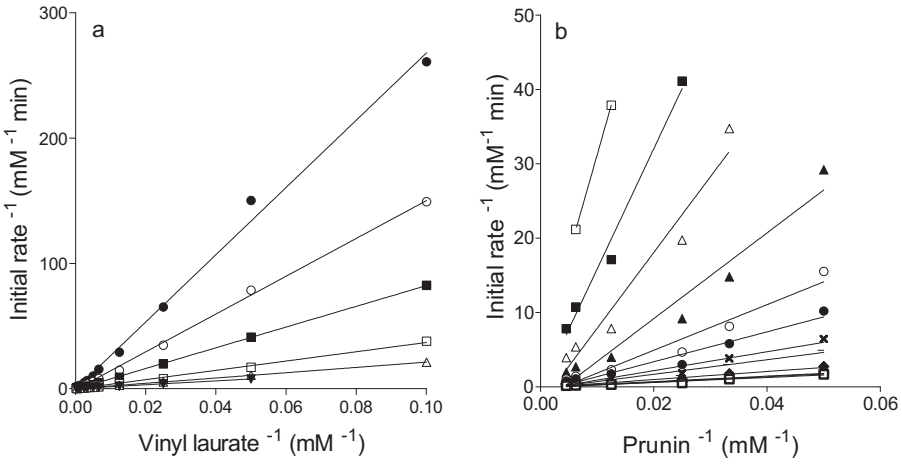


Fig. 3. Lineweaver–Burk plot of reciprocal initial reaction rates versus: (a) reciprocal vinyl laurate concentrations at fixed prunin concentrations (mM): ● 20, ○ 30, 40 mM, □ 80 mM, △ 160 and ▼ 220 and (b) reciprocal prunin concentrations at fixed vinyl laurate concentrations (mM): □ 10, 20, △ 40, ▲ 80, ○ 150, ● 200, × 300, ♣ 400, ♦ 800, ◊ 1600, ▼ 3000 and □ 4000.

The differentiation among the mechanisms associated with Bi Bi systems involves the particular dependence of the apparent parameters with the substrate concentration that is maintained constant [20]. Table 2 shows the behaviour that each kinetic model must perform for K^{ap}, V^{ap}_{max} and the ratio $[V^{ap}_{max}/K^{ap}]$. Fig. 4 shows the experimental values obtained for K^{ap}, V^{ap}_{max} and $[V^{ap}_{max}/K^{ap}]$.

The two mechanisms presented in Table 2 have a hyperbolic dependence between V^{ap}_{max} and the substrate concentration that was kept constant, which is in agreement with the dependence found experimentally. However, the profile experimentally obtained for K^{ap} and $[V^{ap}_{max}/K^{ap}]$ only agreed with the ordered Bi Bi mechanism (K^{ap} decrease with increasing in both P and VL concentration and $[V^{ap}_{max}/K^{ap}]$ had a hyperbolic dependence against the concentration of both substrates). Therefore, the ordered Bi Bi mechanism was chosen and the Ping Pong mechanism was ruled out. Our result was supported, moreover, by the Lineweaver–Burk representations in which the profiles were in agreement with an ordered Bi Bi mechanism (Fig. 3).

The values of the constants associated with the ordered Bi Bi mechanism were obtained through non-lineal regressions of Fig. 4. The kinetic equation that best fits the synthesis of prunin 6″-*O*-laurate using P and VL working with prunin both solubilised and colloidal in acetone catalysed by Novozym 435 at 50 °C is as follows:

$$v = \frac{18\ \text{mM}\ \text{min}^{-1}\ \ [\text{VL}][\text{P}]}{3.84\times10^{5}\ \text{mM}^2 + 305\ \text{mM}\ \ [\text{VL}] + 366\ \text{mM}\ \ [\text{P}] + [\text{VL}][\text{P}]}$$

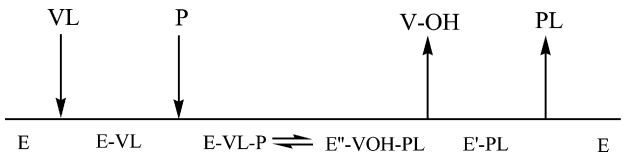
where 18 ± 2 mM min^{-1} is V_{max}, 305 ± 89 mM is K_{mP}, 366 ± 62 mM is K_{mVL} and 3.84×105 mM^2 is the product between K_{VL} (1259 ± 88 mM) and K_{mP}.

The curve fittings upon the experimental data employing this equation is shown in Fig. 5a and b. The experimental initial rates were plotted against the calculated ones (Fig. 5c), obtaining an R^2 of 0.993 and a slope of 0.97 ± 0.01 ($n = 72$). It can be clearly noted that the final kinetic equation is largely consistent with the experimental data, beyond than the systems had been performed using prunin both in solution or colloidal. It is interesting to mention that the equation also describes the behaviour for a solvent-free system, i.e. when vinyl laurate is used as the solvent (curve of [VL] = 4000 mM) up to 220 mM of prunin.

It should be emphasised that although working with colloidal prunin the reaction rates did not follow the same trend that the system with prunin in suspension. Moreover, the behaviour was similar to the systems with prunin completely solubilised. This behaviour probably was due to prunin in colloidal state was available for the enzyme, which surely did not occur when the prunin concentration was 300 mM or higher. In that case, prunin immediately precipitated as a solid, separating itself from the liquid phase. Moreover, the fastest initial rate was obtained with colloidal prunin (220 mM prunin and VL 3000 mM or higher) when the reaction took place at a rate as high as 0.12 mM min^{-1} mg$_{enzyme}^{-1}$ (4.4 kg$_{LP}$ h^{-1} L^{-1} g$_{Enzyme}^{-1}$).

Both, Ping Pong and ordered mechanisms, have been indistinctively reported for lipase-catalysed Bi Bi reactions, but most of the authors assume a Ping Pong mechanism. In some cases it was found that inhibition was produced when an elevated concentration of one of the substrates were used. In addition, enzymes often follow different kinetics for the same type of reaction, according to the intervening substrates. For example, Yadav et al. studied the behaviour of Novozym 435 in several reactions of synthesis and they found Ping Pong mechanisms for the synthesis of perlauric acid [21] and butyl isobutyrate, with alcohol inhibition only in the second case [22]. The same authors also reported that the synthesis of octyl acetate [23], citronellol laurate [24] and cinnamyl laurate [25] followed an ordered mechanism with the formation of a dead-end complex only for the last two products.

The current study determined that synthesis of the flavonoid ester prunin 6″-*O*-laurate (PL) in acetone using prunin as alcohol and vinyl laurate (VL) as acyl group donor catalysed by Novozym 435 at 50 °C with prunin concentration up to 220 mM agrees with an ordered mechanism without inhibition. According to this mechanism lipase (E) reacts first with VL to produce an acyl–enzyme complex (E–VL). Then, this complex must bind to the alcohol prunin (P) to form a ternary complex (E–VL–P). This ternary complex isomerises to another ternary complex (E′-VOH-PL). Then, vinyl alcohol and prunin 6″-*O*-laurate could be released in stepwise. The Cleland representation of the proposed mechanism would be as follows:



There exists a mechanism that is indistinguishable from the ordered mechanism when no inhibition occurs. This mechanism proposed by Theorell and Chance [26] establishes that the ordered Bi Bi mechanism can take place without the formation of ternary

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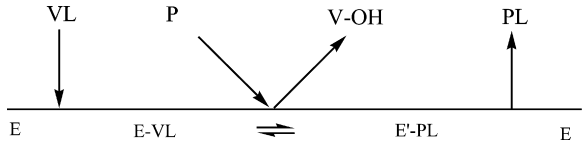
Table 2
Apparent parameters (V_{max}^{ap} and K^{ap}) and their ratio (V_{max}^{ap}/K^{ap}) for each possible kinetic model as a function of each constant substrate concentration.

Mechanism	Rearranged equation		V_{max}^{ap}/K^{ap}	
	Prunin constant concentration	Vinyl laurate constant concentration	Prunin constant concentration	Vinyl laurate constant concentration
Ordered	$V = \frac{\{(V_{max}[P])/(K_{mP}+[P])\}[VL]}{\{(K_{mP}K_{VL}+K_{VL}[P])/(K_{mP}+[P])\}+[VL]}$	$V = \frac{\{(V_{max}[VL])/(K_{mVL}+[VL])\}[P]}{\{(K_{mP}K_{VL}+K_{mP}[VL])/(K_{mVL}+[VL])\}+[P]}$	$\frac{\{V_{max}/K_{mVL}\}[P]}{\{(K_{mP}K_{VL})/K_{mVL}\}+[P]}$	$\frac{\{V_{max}/K_{mP}\}[VL]}{K_{VL}+[VL]}$
Ping Pong	$V = \frac{\{(V_{max}[P])/(K_{mP}+[P])\}[VL]}{\{(K_{mVL}[P])(K_{mP}+[P])\}+[VL]}$	$V = \frac{\{(V_{max}[VL])/(K_{mVL}+[VL])\}[P]}{\{(K_{mP}[VL])/(K_{mVL}+[VL])\}+[P]}$	V_{max}/K_{mVL}	V_{max}/K_{mP}

complexes. The Cleland representation of this mechanism is as follows:



The ordered mechanism is reduced to the Theorell–Chance mechanism if the concentration of the ternary complexes are essentially zero. This means that the activation energies for the formation, isomerisation and decomposition of ternary complexes proposed in the ordered mechanism are virtually zero. This mechanism has not been yet mentioned for lipases but it has been proposed for other enzymes [27]. In his book, *Enzyme Kinetics*,

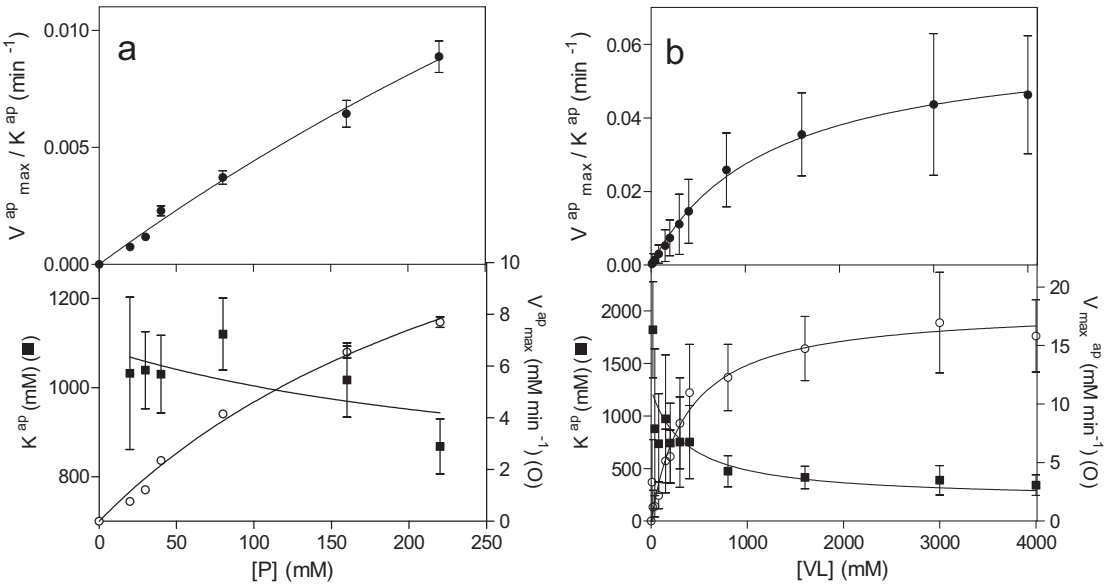


Fig. 4. Apparent Michaelis–Menten parameters (V_{max}^{ap} and K^{ap}) and their ratio (V_{max}^{ap}/K^{ap}) for the synthesis of prunin laurate depending on the concentration of (a) vinyl laurate and (b) the concentration of prunin. Each point is the mean of two values ± standard deviation.

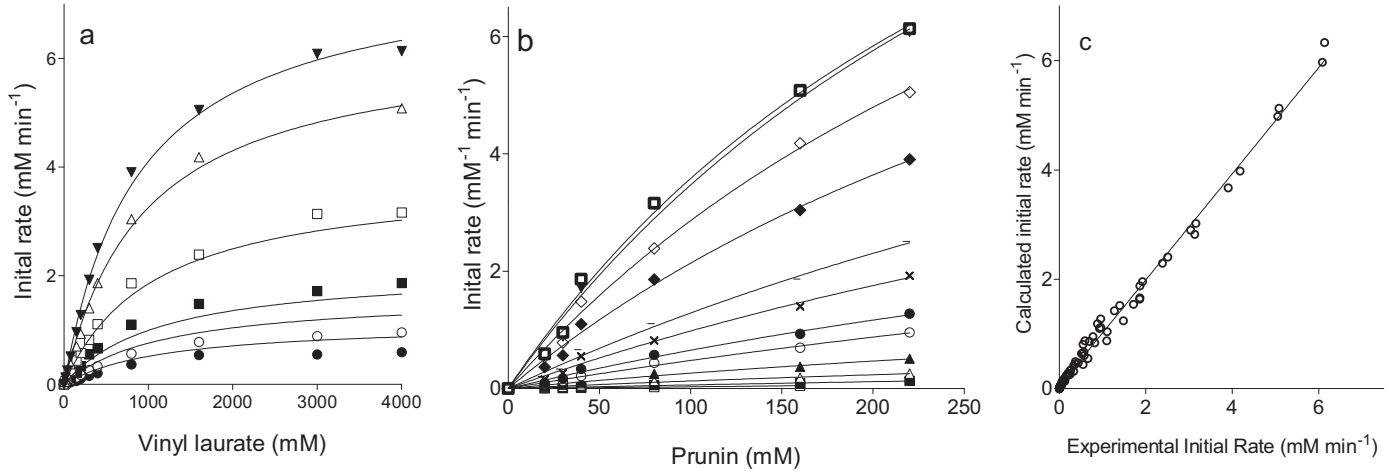


Fig. 5. (a) Dependence of initial rate on vinyl laurate concentration at several fixed pruninconcentrations. P (mM): ● 20, ○ 30, 40, □ 80, △ 160 and ▼ 220. (b) Dependence of initia rate on prunin concentration at several fixed vinyl laurate concentrations. VL (mM): □ 10, 20, △ 40, ▲ 80, ○ 150, ● 200, × 300, ♣ 400, ♦ 800, ◊ 1600, ▼ 3000 and □ 4000. Solid lines were plotted with the final kinetic equation. (c) Comparison of experimental and calculated rates (slope 0.97 and R^2 0.993, $n = 72$).

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Segel believes that it is unlikely that exists a "real" Theorell–Chance mechanism [28].

4. Conclusions

The current study determined that the synthesis of prunin 6″-*O*-laurate can be performed efficiently in media with prunin both, completely solubilised or forming colloids. The kinetic model that best fit the experimental data in these conditions was an ordered Bi Bi mechanism without inhibition. The equation had a strong fitting with the experimental data and, moreover, it was applicable to a solvent-free system.

Acknowledgments

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