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Role of apparent pK_a of carboxylic acids in lipase-catalyzed esterifications in biphasic systems

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ABSTRACT

Lipase-catalyzed esterifications in biphasic media (heptane–water, 1:1) were conducted by using *Thermomyces lanuginosus* lipase (TLL) as biocatalyst. Different carboxylic acids (from acetic to lauric) were thus esterified with 1-butanol at different pH values (2–10). For all carboxylic acids tested, pH optima for the enzymatic esterifications were ca. 3–5, in clear agreement with previous literature, and quite different from optima pH of TLL in hydrolytic reactions (8–11). Interestingly, the interval of pH in which TLL was active in esterifications, varied markedly depending on the carboxylic acid. Thus, for long-chain acids (i.e. lauric), such pH interval was much wider than for short-chain carboxylic acids (i.e. acetic). To understand this effect, simple pK_a values of carboxylic acids, retrieved from open literature, are not sufficient, since those values are measured in pure water. When a second phase is added, aspects related to partition coefficient of each carboxylic acid need to be considered as well, thus leading to the apparent pK_a concept. Herein we performed theoretical calculations to obtain such app pK_a of each carboxylic acid. When such calculations were compared with the pH interval, a clear correlation was observed. Overall, results confirm that lipases accept only the protonated form of the carboxylic acid (R–COOH) during esterifications in biphasic media.

1. Introduction

Lipases are well-known biocatalysts, very useful for the production of a wide range of compounds under mild reaction conditions. Many processes involving the use of lipases have been implemented at industrial scale [1–8]. An important asset of these enzymes is their capability to efficiently catalyze reactions also in non-aqueous conditions – organic solvents, solvent-free systems, ionic liquids, and supercritical CO_2 – which obviously confers these biocatalysts a superior scope of applications [1–11]. In addition, the use of lipases to conduct synthetic reactions in biphasic media (i.e. esterifications), and by means of reverse micelles (which are actually biphasic media), has been reported as well [12–17].

Despite this ample use of lipases in biocatalysis, limited attention has been paid, however, to the influence of pH in lipase-catalyzed esterifications in biphasic media. Apart from some articles focusing on the electrostatic contribution to hydrolytic performances [18–22], to the best of our knowledge no systematic studies regarding the influence of the pH in hydrolase-catalyzed esterifications in biphasic media were published until 2005, when the innovative work of Buthe et al. was disclosed [23]. In that article, it was reported that pH optima for lipase-catalyzed esterifications differed widely from values observed when hydrolytic reactions were conducted. Buthe et al. showed that pH optima for lipase-catalyzed esterifications in two-phase media were around 3–5 [23]. In virtue of the results reported, it was formulated then the hypothesis that lipases would only accept the protonated form of the carboxylic acid. Therefore, reactions at pH values lower than the pK_a of the carboxylic acid would show higher enzymatic synthetic performances, as more *actual* substrate molecules would be available for the lipase [23]. We were particularly interested in exploring this pH-influence concept when different carboxylic acids were applied. In this paper part of that research is disclosed.

2. Experimental

2.1. Chemicals and biocatalyst

Heptane, carboxylic acids and alcohols were all obtained from Sigma–Aldrich and Fluka, and were used without further purification. *Thermomyces lanuginosus* lipase (TLL) was purchased from Novozymes: Lipozyme TL® 100L (LAP 40001), 100 KLU/g. 1 KLU (kilo-lipase-unit) is the amount of enzyme that liberates 1 mmol/min of titratable butyric acid from tributyrin.

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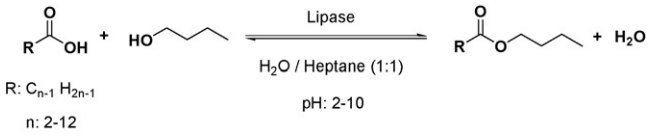
2.2. Enzymatic reactions.

The following procedure was used to evaluate the effect of pH and acid type on esterification reaction rate. 25 mL of distilled water was mixed with 25 mL of heptane. Subsequently, alcohol (300 mM) and carboxylic acid (300 mM) were added. During experiments with lauric acid, 600 mM of both acid and alcohol were added. For molar calculations, one phase (25 mL) was considered. Previous experiments in our laboratory showed that at those reaction conditions a linear relationship between substrate concentration and enzymatic rate was found. The pH was adjusted to the desired value by adding concentrated HCl or NaOH. The system was stirred vigorously until the desired pH was reached and was constant, meaning that the partition of both substrates in the biphasic system had reached the equilibrium. Significant variations of pH (>0.2 units) were not observed during the enzymatic reactions, since only initial rates were measured, and at that time range conversions were low (<10%). Reactions were carried out at 25 °C, and in the pH interval from 2 to 10. The reaction was started by addition of 200 μL of TLL. Aliquots of the organic phase were taken during the reaction. For acetic acid, propionic acid and hexanoic acid, the reaction system contained two distinct separate phases, whereas for octanoic and lauric acid, an emulsified system was observed. Initial rates (up to 6 h reaction) were measured, and data normalized separately in each pH case.

2.3. Analytical methods

Enzymatic reactions were followed by GC analysis, using hexadecane as external standard for the calculation of the conversion. To this end, calibration curves of products (butyl esters) were performed (range 0–100 mM) using pentane as solvent, with a fixed concentration of hexadecane (100 mM). Thus, a "Response Factor", that is [area butyl ester]/[area 100 mM hexadecane] was calculated, and plotted in a graphic (response factor *vs.* [butyl ester]). For measurements of real samples, conversions were obtained by calculating that response factor in each case, and intrapolating values in calibration curve. For the GC analysis, an initial column temperature of 40 °C was set. This temperature was increased 75 °C/min until 230 °C. The injector temperature was 275 °C, and a constant column flow of 1 mL/min. Detector temperature was 290 °C, using helium as carrier gas. Samples of 0.1 μL were analyzed.



Scheme 1. General scheme of the TLL-lipase-catalyzed esterification performed in biphasic system.

3. Results and discussion

We studied lipase-catalyzed esterifications in biphasic systems (heptane–water) by using butanol and different aliphatic carboxylic acids – from acetic to lauric acid – as substrates, and operating at different pH levels (Scheme 1). Lipase from *Thermomyces lanuginosus* (TLL) was chosen as model biocatalyst in virtue of its wide difference in pH optima between hydrolytic and synthetic approaches (8–11 *vs.* 3–5 respectively) [23].

The results of our investigations about enzymatic esterifications in biphasic systems using different carboxylic acids are summarized in Fig. 1.

As observed in Fig. 1, all carboxylic acids tested showed an analogous pH-optima profile, displaying a maximum of activity of TLL at a pH of ca. 3–5. This is in clear agreement with data reported by previously [23]. Remarkably, from Fig. 1 another interesting fact can be observed: the pH interval of the enzymatic activity profile varies with the chain length of the carboxylic acid used in each case. In this respect, when lauric acid was tested as substrate, it was possible to observe TLL-catalyzed esterifications up to a pH

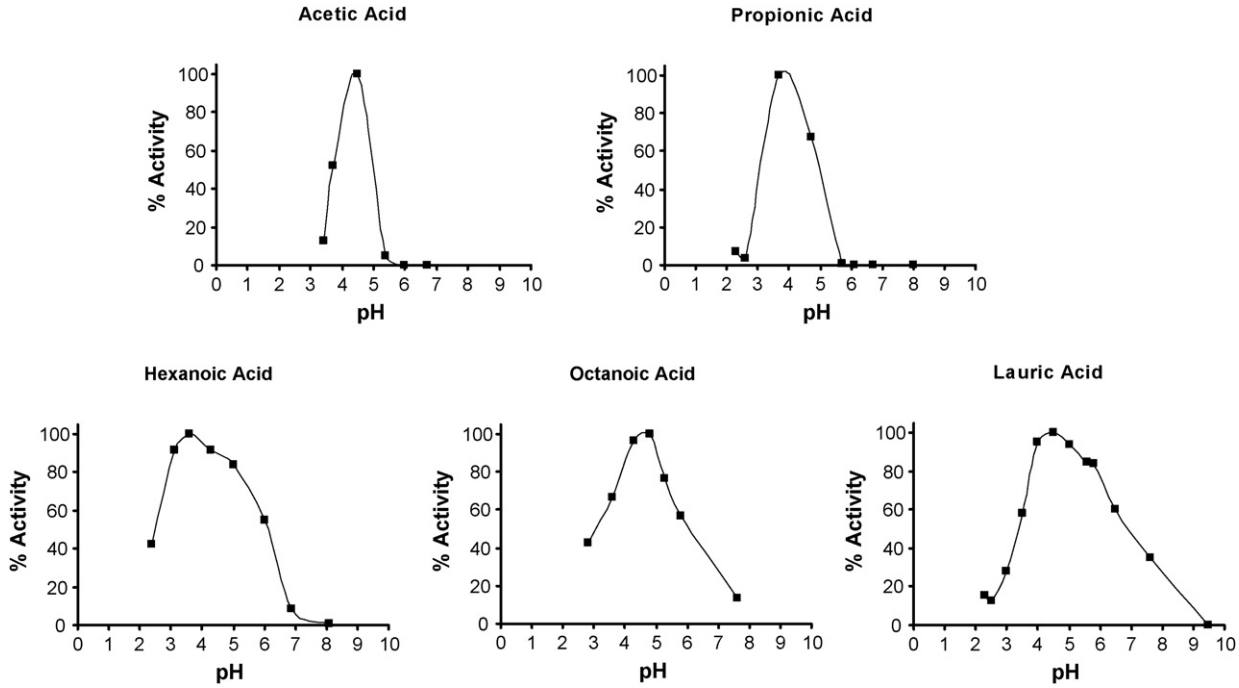


Fig. 1. Active pH-profile in the TLL-catalyzed esterification of aliphatic carboxylic acids (acetic to lauric) with butanol in biphasic media. Activity was determined by GC, as mmol ester formed L^{-1} h^{-1}. The % activity, is the percentage of maximum esterification activity observed for that specific acid. Each acyl donor displayed different kinetic patterns, and graphics were normalized in all cases. Highest rate values (mM/h) of each carboxylic acid were: acetic, 0.004; propionic, 0.042; hexanoic, 0.043; octanoic, 0.030 (separated two-phases), and 2.3 (emulsion); lauric, 0.68 (emulsion). Rates were measured up to 6 h reaction, and <10% conversions were achieved in all cases.

of approximately 8–9, though at low conversion rates. These latter results regarding lauric acid correlate well with publications in which lipase-catalyzed processes in reverse micelles (which are actually a type of biphasic media) were conducted at a neutral pH (pH 7) [13,14]. Moreover, a recent work reported by Fernandes et al. [24], who studied TLL-catalyzed esterifications by entrapping the biocatalysts in such reverse micelles, showed that, out of the different carboxylic acids evaluated as acyl donors, only lauric acid appeared to be a proper substrate for the enzyme to produce alkyl laurate at pH 8, whereas smaller aliphatic carboxylic acids were not esterified by TLL at this pH [24]. In addition, those authors found an optimum pH of 5.6 for this esterification with lauric acid in biphasic (reverse micelles) media. That pH of 5.6 has also been recently used for hydrolase-catalyzed esterification of oleic acid in biphasic media as well, suggesting that this pH interval is optimum for other fatty acids (see below in this paper) [25]. Notably, and contrary to the performance of the lauric acid, the pH interval of enzymatic activity for acetic acid was rather narrow (active pH of ca. 3–5, Fig. 1).

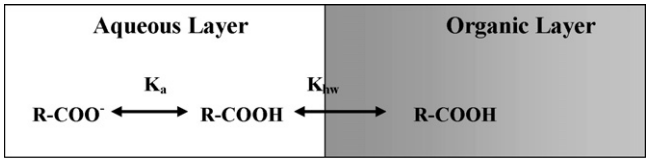
In their inspiring work, Buthe et al. [23] suggested that only the protonated form of the carboxylic acid (R–COOH) would be the actual acyl donor for the lipase. Therefore, those pH values below pK_a would be the most suitable ones for synthetic reactions. However, pK_a values reported in literature for aliphatic carboxylic acids are in the range of 4.74–4.90 (acetic to lauric). Obviously, those values cannot fully explain the behaviour herein observed. For instance, lauric acid is accepted as substrate at pH values of ca. 8, which is ca. 3 pH units higher than its reported pK_a (Fig. 1). To be able to understand these intriguing results, we reasoned that pK_a values reported in literature are measured in water, a single phase system, instead of the biphasic heptane–water system herein used. The pK_a values reported in literature are thus markedly different from the so-called apparent pK_a (app pK_a), which is influenced by the distribution of the acid over the two phases existing in a biphasic media (in our case, water–heptane). Therefore, the app pK_a is related to the pK_a, as obtained in open literature from single phase water systems [26], as well as to K_{hw} (the partition coefficient of acids in heptane and water $K_{hw} = [Ac]_h/[Ac]_w$) (Fig. 2).

From Fig. 2, apparent pK_a can easily be obtained by making use of Eq. (1):

$$\text{app p}K_a = \text{p}K_a + \log\left(1 + K_{hw} \times \frac{V_h}{V_w}\right) \quad (1)$$

Since, in our experiments equal volumes of heptane and water were used, Eq. (1) can be simplified to Eq. (2):

$$\text{app p}K_a = \text{p}K_a + \log(1 + K_{hw}) \quad (2)$$



$$RCOOH + H_2O \xleftrightarrow{K_a} RCOO^- + H_3O^+$$

$$pK_a = -\left(\frac{RCOO^- \; H_3O^+}{RCOOH}\right) = -\left(\frac{RCOO^-}{RCOOH}\right) + pH$$

$$RCOOH_{\,heptane} \xleftrightarrow{K_{hw}} RCOOH_{\,water}$$

$$K_{hw} = \left(\frac{RCOOH_{\,heptane}}{RCOOH_{\,water}}\right)$$

Fig. 2. Carboxylic acid partition in two phases. Significance of (apparent) pK_a depending on the pH.

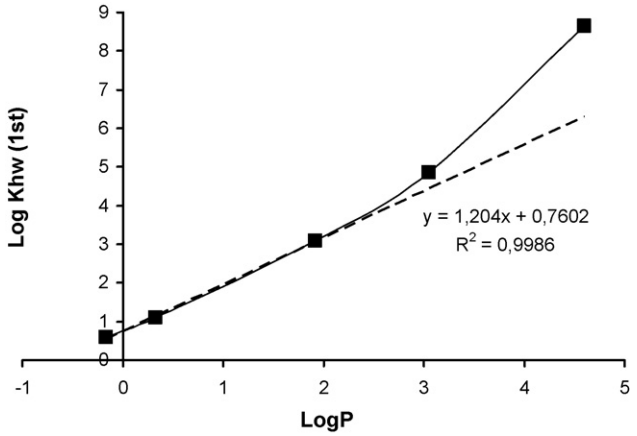


Fig. 3. Correlation between log P (partition coefficient octanol–water) [26], and log(K_{hw} (1st)) (partition coefficient heptane–water, modelled) of different carboxylic acids (from acetic to lauric).

Eq. (2) means that long-chain aliphatic carboxylic acids will actually have a higher app pK_a than short chain ones, since their lipophilicity will lead to increased solubility in the organic phase and thus a larger partition coefficient K_{hw}. Conclusively, a stronger alkalinity (higher pH) will be needed to dissociate the total acid fraction. Thus, a correlation between pH interval of lipase activity and these app pK_a's should be found.

To test this hypothesis for our reaction system, true pK_a values of the carboxylic acids (in water) were retrieved from literature [26]. Subsequently, two approaches were used to obtain values for the partition coefficient K_{hw}. In the first approach the K_{hw} values were assessed from liquid–liquid equilibrium (LLE) calculations with *Aspenplus*, using the UNIFAC model (results of these calculations are shown in Table 1, column 1, for log(K_{hw} (1st)). In this approach, it is important to consider that this theoretical assessment assumes that acid molecules are *either* fully dissolved in the organic phase *or* in the aqueous phase. Therefore, partial partitioning, where part of the molecule resides in the organic layer and the other part in the aqueous layer – like emulsifiers, e.g. octanoic acid and lauric acid do – is not taken into account. Consequently, due to this "simplification" for these longer chain carboxylic acids, extremely high activity coefficients were obtained in our model calculations, leading to overprediction of K_{hw} and thus of the app pK_a as well. Therefore, to improve the assessment of these values for longer chain carboxylic acids a second approach was used. In this approach log(K_{hw}) was obtained based on the assumption that for all carboxylic acids used, there would be a linear relation between log(K_{hw}) and the well known and documented log P, the partition coefficient between n-octanol and water (Fig. 3).

As observed in Fig. 3, for short-chain carboxylic acids this linear dependency between log P and our log K_{hw} (1st) approach is indeed found, and can be described by Eq. (3).

$$\log(K_{hw}(1st)) = 1.204 \times \log P + 0.7602 \quad (3)$$

However, for longer chain carboxylic acids (octanoic acid and lauric acid) the value for log(K_{hw} (1st)) deviates from the linear dependency – compared to log P – and higher values than expected are found on the basis of the correlation. As explained above, this may be because our *Aspenplus*-UNIFAC model assessment for log(K_{hw} (1st)) assumes that acids are *either* in one phase *or* in the other, and thus, important surfactant-like behaviours (partial partitioning), are not considered. To overcome this issue, in our second approach we therefore calculated log(K_{hw} (2nd)) values according to the linear dependency shown in Eq. (3).

Table 1
Theoretical calculations of the app pK_a of different carboxylic acids in a two-phase system (water–heptane).

Acid	Input			Output
	1	2	3	4
	"True" pK_a[a]	log(K_{hw} (1st))[b]	log(K_{hw} (2nd))[c]	App pK_a[d]
Acetic (CH_3COOH)	4.756	0.597	0.556	5.4
Propionic (C_2H_5COOH)	4.874	1.103	1.158	6.1
Hexanoic ($C_5H_{11}COOH$)	4.849	3.085	3.072	7.9
Octanoic ($C_7H_{15}COOH$)	4.895	4.855	4.432	9.3
Lauric ($C_{11}H_{23}COOH$)	4.900	8.640	6.299	11.2

[a] Taken from open literature [26].
[b] Values resulting from the first theoretical calculation of the partition coefficient, based on *Aspenplus*-UNIFAC.
[c] Theoretical reassessed calculation, based on Eq. (3).
[d] Value obtained from this second theoretical assessment, using Eq. (2).

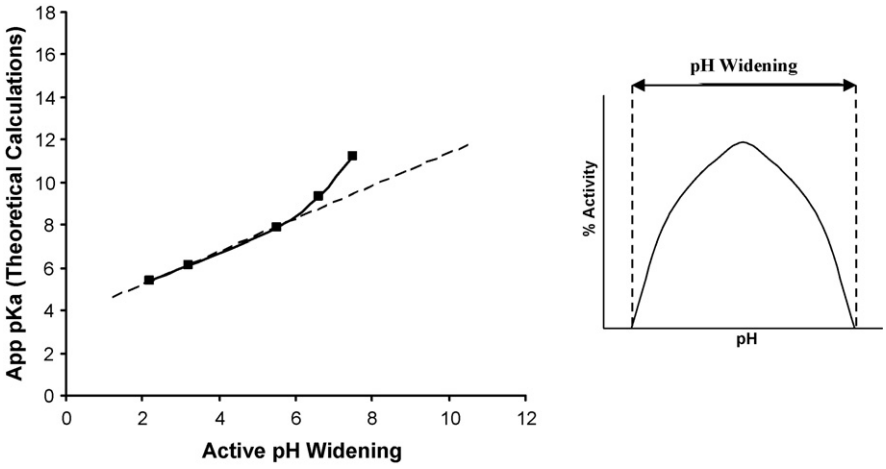


Fig. 4. The active pH interval related to app pK_a determined by theoretical calculations based on pK_a of the aliphatic carboxylic acid in water, as well as the partitioning coefficient of the acid in heptane and water (K_{hw}).

Later on, based on the estimation of log(K_{hw} (2nd)) the app pK_a of each carboxylic acid was then calculated from Eqs. (1) and (2). In Table 1 all relevant data of this study are summarized.

From Table 1 it can be concluded that app pK_a's of different aliphatic carboxylic acids differ considerably. Furthermore, the values will vary even for the same acid, if physico-chemical conditions of the environment (and/or the type (and volume) of organic solvent) are changed [27,28], as these conditions affect the partition coefficient.

Interestingly, a correlation is observed between the pH reaction interval (pH widening), in which TLL-catalyzed esterification occurs for the different carboxylic acids (Fig. 1), and the app pK_a values (Fig. 4).

The app pK_a can therefore be considered as an important factor for explaining the reported lipase-catalyzed biocatalytic performances in biphasic media. Furthermore, based on the correlation obtained, pH ranges for which reactivity of the enzyme is expected can be determined. As observed in Fig. 3, for longer chain aliphatic carboxylic acids still a deviation from a linear relation between pH interval and app pK_a is found. Possibly, the use of a more sophisticated theoretical model, in which other parameters are considered as well, might lead to better correlations when long-chain carboxylic acids are evaluated.

It is important to note that this pH interval (for enzymatic esterifications) is an *absolute* measurement. That means that we are evaluating if a lipase is *either* active *or* not at a certain pH when a certain carboxylic acid is added, in certain (biphasic) reaction conditions. Therefore, specific parameters that each lipase will have towards each substrate/reaction system – that is, concentration, potential inhibition, K_m, K_{cat}, conversion at equilibria, etc., – will certainly change the performance of the enzyme, but will not influence the pH interval, since this value *simply* indicates whether or not a carboxylic acid is present as active substrate for the lipase (R–COOH). Likewise, this consideration can also have value in (biochemical) aqueous systems in which a non-soluble acid is added. Since that set-up is actually a biphasic medium, it is expected that the apparent pK_a will also play a role in results observed.

4. Conclusions

The apparent pK_a of carboxylic acids is an important parameter to understand hydrolase-based reactivities in biphasic media. Our work fully confirms the hypothesis made by Buthe et al. [23], stating that only protonated forms of the carboxylic acids are actual substrates for the enzymes. The herein presented correlation between pH interval and app pK_a can be used to assess whether at a specific pH a desired lipase-catalyzed esterification in a biphasic medium will occur or not. The same type of reaction may thus have a narrower or wider active pH interval simply depending on the carboxylic acid used. Traditionally, the studies aiming to understand hydrolase-catalyzed behaviours are based on changes produced in the protonation states of amino acidic residues present in the active site at different pH values. Our results show that, besides those clearly important issues, the nature of the reagents and solvents used influences the enzymatic performance to a large extent as well.

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