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Selectivity of lipases and esterases towards phenol esters

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Abstract

The selectivity of 28 lipases and esterases in the hydrolysis of butanoates of *o*-, *m*- or *p*-substituted phenols was investigated in a microtiter-plate format. The phenols released during enzyme-catalyzed hydrolysis were converted in situ with Gibbs' reagent to form a blue indophenol complex, which was quantified spectrophotometrically at 600 nm. Substantial differences in rates were found, which exhibits that the type and position of the substituent at the alkyl group has a strong influence on the selectivity of the enzymes. For various enzymes, the *p*-nitro derivative was the best substrate, whereas for other enzymes the *m*-Cl-derivative was preferentially hydrolyzed. Analysis of the data using the Hammett equation showed that sometimes the observed changes followed a predictable trend, but in several cases the result is very unexpected.

Keywords: Lipase; Gibbs' reagent; Hammett equation; Esterase; Phenol esters

1. Introduction

Lipases are an important class of biocatalysts, which are frequently used in lipid modification [1] and in organic synthesis [2]. Enzymes in this class have been shown to be 1,3-regioselective for triglycerides, selective for fatty acid chain length and degree of fatty acid saturation [3]. *p*-Nitrophenyl esters are ideal substrates to assay lipase and esterase activity, as the *p*-nitrophenolate released can be easily quantified spectrophotometrically at 410 nm. Less activated methyl- or ethyl-esters are generally worse substrates and *tert*-butyl esters are hardly cleaved at all. Initially, we aimed to develop a lipase assay, which would facilitate rapid discrimination of the fatty acid chain-length selectivity of an enzyme. The basic concept of the spectrophotometric assay is shown in Scheme 1. One fatty acid (e.g., myristic acid) is used as *p*-nitrophenyl ester, a second fatty acid (e.g., butanoic acid) is esterified with phenol. Upon incubation of these esters in the presence of a lipase, both esters should be hydrolyzed at rates dependent on the enzyme's selectivity for one over the other ester substrate. Released *p*-nitrophenol can be directly quantified spectrophotometrically at 410 nm. Released phenol is reacted with Gibbs' reagent (2,6-dichloroquinone-4-chloroimide) [4,5] present in the reaction mixture and the blue indophenol complex formed is then quantified at 600 nm. The ratio of colored products would provide a measurement of the selectivity of the enzyme i.e. a selectivity ratio.

During development of this assay it was observed that the enzymes did not only discriminate for the fatty acid chain-length, but also for the alkyl group. For example, when phenol and *p*-nitrophenol butanoates were co-incubated in the presence of a range of hydrolases, the expectation was, that a similar selectivity ratio (rate of *p*-nitrophenolate formation over

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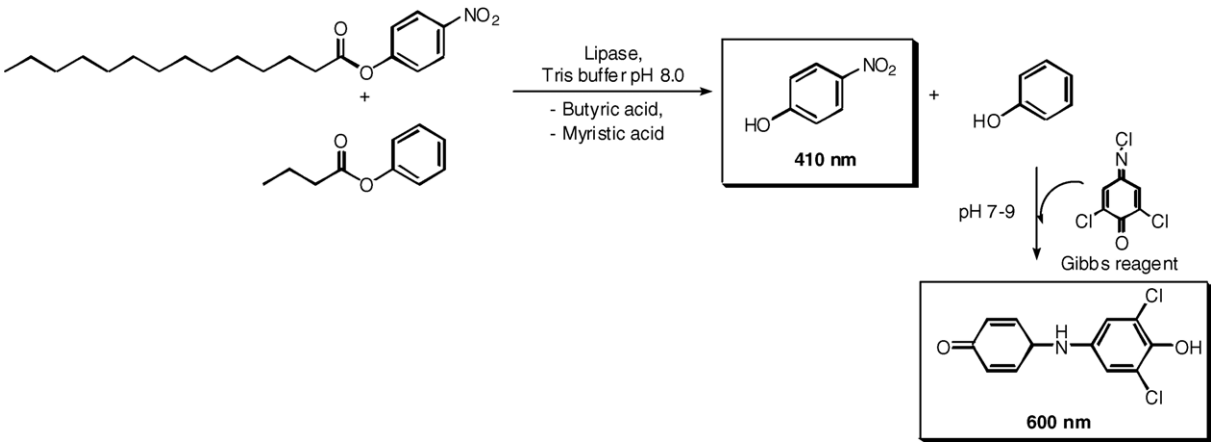


Scheme 1. Proposed assay format to determine lipase selectivity towards different fatty acids. The example shows *p*-nitrophenyl tetradecanoate and phenyl butanoate. *p*-Nitrophenol is quantified at 410 nm. Phenol released reacts readily under alkaline conditions with Gibbs' reagent (2,6-dichloroquinone-4-chloroimide) to form a blue indophenol derivative, which is measured at 600 nm.

phenol formation) would be observed for all the enzymes, as the fatty acid chain-length was the same for both substrates. Surprisingly, it was found that some enzymes showed higher activity towards the phenyl ester, while others preferentially cleaved the *p*-nitrophenyl ester (data not shown).

In light of the above finding, we were interested in exploring the selectivities of the different enzymes with respect to a series of leaving alcohols. We therefore synthesized a range of phenol esters bearing different substituents at the *o*-, or *m*-position of phenol and investigated the selectivity of a collection of hydrolases towards these compounds (Scheme 2). The results obtained were then analyzed using the Hammett equation.

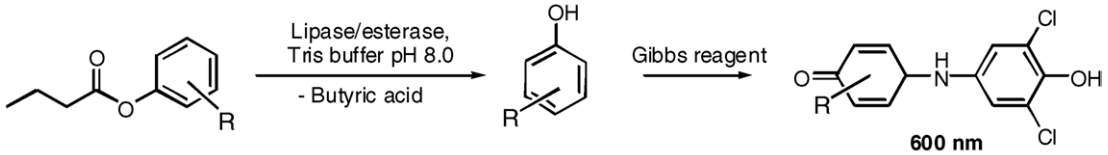
The presence of substituents in an aryl system can stabilize or destabilize the accumulation of charge in the transition state of a chemical reaction. The Hammett equation is applied to evaluate the inductive effects due to the introduction of *m*- or *p*-substituents on an aryl system on any desired property of the reaction:

$$\log K - \log K_0 = \sigma\rho$$

Positive values of the reaction constant ρ result from an enhancement of the reaction rate by electron withdrawing groups, whereas electron donating groups yield negative ρ values. The magnitude of the reaction constant is related to the sensitivity of the reaction rate towards the electronic effect caused by the presence of the substituent on the aromatic ring [6]. The sign and magnitude of the reaction constant are commonly visualized graphically by means of the Hammett plot, i.e. a graphic representation of the property under investigation versus the Hammett constant for every substituent, σ. Such analysis has been also applied to enzyme kinetics [7] to evaluate the effect of substituents on properties such as enantioselectivity [8,9], reaction rate [10] and binding constants [11,12].

2. Materials and methods

All chemicals were purchased from Fluka and Sigma at the highest purity available. Commercial enzymes used: lipase B from *Candida antarctica* (CAL-B, Novozymes), lipase from *Rhizopus oryzae* (ROL, Amano F-AP 15), lipase from *Burkholderia cepacia* (BCL, Amano PS), lipase from *Candida rugosa* (CRL, Amano AY), pig liver esterase (PLE, Roche). Esterase from *Pseudomonas fluorescens* (PFE) and esterase from *Bacillus subtilis* (BsubpNBE) were recombinantly expressed in *E. coli* BL21 (DE3) [F^- *omp*T *hsd*S_B($r_B^-m_B$) *gal dcm*(DE3)] [13] and used as lyophilized crude cell lysates. All other enzymes (DVSA1-21) were produced recombinantly and supplied as lyophilized crude cell lysates by Diversa Corp. (San Diego, USA). These enzymes were discovered from environmental DNA libraries prepared



Scheme 2. Model reaction and phenol derivatives used.

from a vast array of ecological niches and biotopes that span the globe. The libraries were created using methods described previously [14–16]. Owing to this method of enzyme discovery the exact nature of source organism cannot be precisely determined. However, sequence based analyses of Diversa libraries [14] and of the lipases discussed here (data not shown) indicate that the DVSA enzymes are most likely bacterial or archaeal in origin.

2.1. Synthesis of phenyl esters *1–9*

Butanoyl chloride (50 mM) and phenol (**1**–**9**, 150 mM) were dissolved in 70 ml dichloromethane using $ZnCl_2$ (10 g) as catalyst. The mixture was refluxed until thin-layer chromatography indicated no further product formation. The mixture was cooled and poured into 300 ml ice water. After twice extracting with diethyl ether, the combined organic layers were washed twice with sodium carbonate solution and water, and then dried over anhydrous sodium sulfate. Excess phenol was removed under vacuum and the residue was purified by silica gel chromatography (hexane:ethyl acetate, 10:1 or 5:1). Yields—**1**: 48.5%; **2**: 57%; **3**: 33%; **4**: 8%; **5**: 17%; **6**: 38%; **7**: 46%; **8**: 32%; **9**: 21%. Chemical identity of all compounds was confirmed by ^{1}H and ^{13}C NMR spectroscopy.

2.2. Determination of extinction coefficients

Stock solutions of all phenols (100 mM) were prepared in DMSO. Samples were diluted (in DMSO) 1:10, 1:20, 1:50, 1:100, 1:200, 1:500 and 1:1000. Thirty microliter aliquots of each solution were then added to wells of a microtiterplate (MTP) containing Tris buffer (50 mM, pH 7.2–8.0). Except for *p*-nitrophenol, 30 μl of the Gibbs' reagent (stock solution: 20 mM in EtOH) were also added to the MTP. The final volume per well was 250 μl. Samples were then incubated for 10 min at room temperature and the absorption value was determined spectrophotometrically at 410 nm (*p*- and *m*-nitrophenol) or at 600 nm (phenol-Gibbs' adduct). From the data obtained, extinction coefficients were calculated (Table 1). All measurements were made in triplicate.

Table 1
Extinction coefficients determined for *m*- or *p*-nitrophenol (**8**, **10**) (at 410 nm) and the phenol/Gibbs' adducts (**2**–**7**, **9**) (at 600 nm)

Compound	Extinction coefficient (M^{-1})
1	2635
2	12599
3	8473
4	12797
5	9040
6	3143
7	1289
8	184[a]
9	n.d.[b]
10	12447[a]

[a] At 410 nm.
[b] The reaction of compound **9** with Gibbs' reagent was negligible.

2.3. Determination of enzyme selectivity, activity and initial rates

Stock solutions of phenyl esters (5 mM) were prepared in DMSO. The Gibbs' reagent (20 mM) was dissolved in EtOH. These solutions were then mixed 1:1 on a volumetric basis to provide the assay stock solution. Next, 120 μl Tris buffer were added to each well of a microtiterplate followed by enzyme samples (100 μl from a stock solution containing 0.2 mg enzyme/ml). The reactions were started by addition of 30 μl substrate solution from the assay stock solution to each well of the microtiterplate using a 12-channel multipipette. The plate was then incubated in a microtiterplate reader and absorption values were determined by spectrophotometric measurement at 410 nm (*p*- and *m*-nitrophenol) or 600 nm (phenol-Gibbs' adduct) for 10 min at 37 °C with intermittent shaking. From the data, initial rates and specific activities were calculated. All measurements were made in triplicate. Adequate controls with no enzyme extract were run in parallel to each determination. In the case of PFE and BsubpNBE additional controls with an extract from the empty host were run.

2.4. Hammett plot

Initial rates of the enzymatic reactions only with *m*-substituted phenols were considered, since the *o*-effect on reaction rates requires a more complex approach. Phenyl esters were taken as a reference, and thus the log of the ratio of the specific activity for each ester over the activity for the phenyl ester was used to plot against each respective Hammett constant.

3. Results and discussion

First, the absorption spectra of *p*-nitrophenol, and the indophenol compounds were measured in the range 350–750 nm (data not shown). Since the derivatization of phenol with Gibbs' reagent takes place under slightly alkaline conditions but most lipases show highest activity around neutral pH, the derivatization was performed at different pH-values and the extinction coefficients for *p*-nitrophenol and the indophenol compounds were determined. As a compromise, Tris 50 mM pH 8.0 buffer was used for all subsequent assays. The extinction coefficients thus determined (detailed in Table 1) were used to calculate specific activities. The *m*-CN substituted phenol **9**, did not react with Gibbs' reagent, and therefore it was not possible to monitor the hydrolysis of the corresponding butyrate.

Fig. 1 shows the ratio of the specific activity for each phenol ester divided by the maximum activity (expressed as percentage), for seven enzymes. All lipases (CAL-B, ROL, BCL, CRL) and one esterase (PFEI-) show similar "selectivity profiles", that is, approximately a similar distribution of activities on the different substrates, with the *p*-NO_2 and

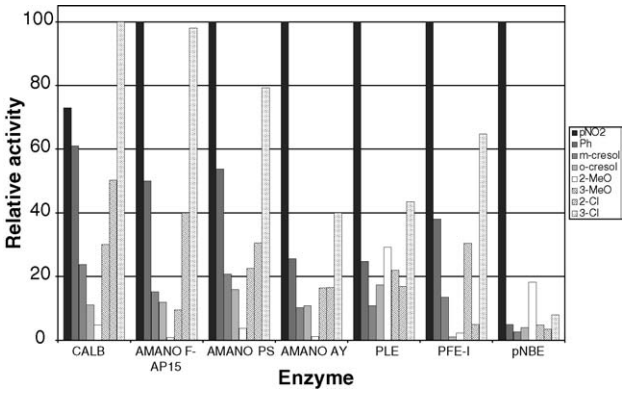


Fig. 1. Relative activity (ratio between specific hydrolytic activity measured with a substituted phenyl ester to the maximum specific activity measured) for seven enzymes. Experimental conditions for the assay are detailed in Section 2. Specific activities ranged from 24 mU/mg to 18.5 U/mg.

the *m*-Cl derivatives yielding the highest activities. The other two esterases (PLE and BsubpNBE) show profiles, which are different to the lipases, but similar when compared to one another. It should also be noted that only for CAL-B, the *p*-nitrophenyl ester was not the best substrate, but the *m*-Cl derivative.

Fig. 2a–c shows the ratio of the specific activity for each phenol ester divided by the maximum activity for 21 Diversa enzymes. Similar selectivity profiles to the four commercial lipases were found for enzymes DVSA7, DVSA8, DVSA9, DVSA10 (Fig. 2b). A different profile in which *o*-Cl provides a better rate than *m*-Cl was found for six of the Diversa enzymes tested, namely, DVSA1, DVSA2, DVSA3, DVSA4, DVSA5 and DVSA6 (Fig. 2a). A third clear pattern can be observed, in which *m*-substitution of the substrate clearly favors enzymatic activity, and this was the case for enzymes DVSA11 and DVSA12 (Fig. 2b). The selectivity profiles of the remaining Diversa enzymes are shown in Fig. 2c.

Hammett analysis of the data established a correlation between enzyme activity and electronic effect of the substituent in the aromatic ring. The Hammett plot for the seven non-Diversa enzymes is shown in Fig. 3. For the group of six enzymes that exhibit positive slopes, values of reaction constant ranged between 0.8 and 1.6. From the sign of the slope, it may be concluded that the inductive effect of the substituents in the *m*-position enhances the hydrolysis reaction. In contrast, in the case of pig liver esterase, there seems to be no electronic effect caused by the different *m*-substituents.

Fig. 4a and b shows the Hammett plot for 19 of the 21 Diversa enzymes. The enzymes show different slopes, from enzymes not influenced by the electronic effect or influenced very little, such as DVSA13, DVSA15, DVSA11, DVSA12 to enzymes DVSA8 and DVSA5, which exhibited the highest slope from the set of 19.

To explain the inductive effect of a differently substituted phenyl ring on the enzyme activity, a closer look at the enzyme mechanism of lipases is necessary. Catalysis takes place in a four-step process ([2], Scheme 3, exemplified using

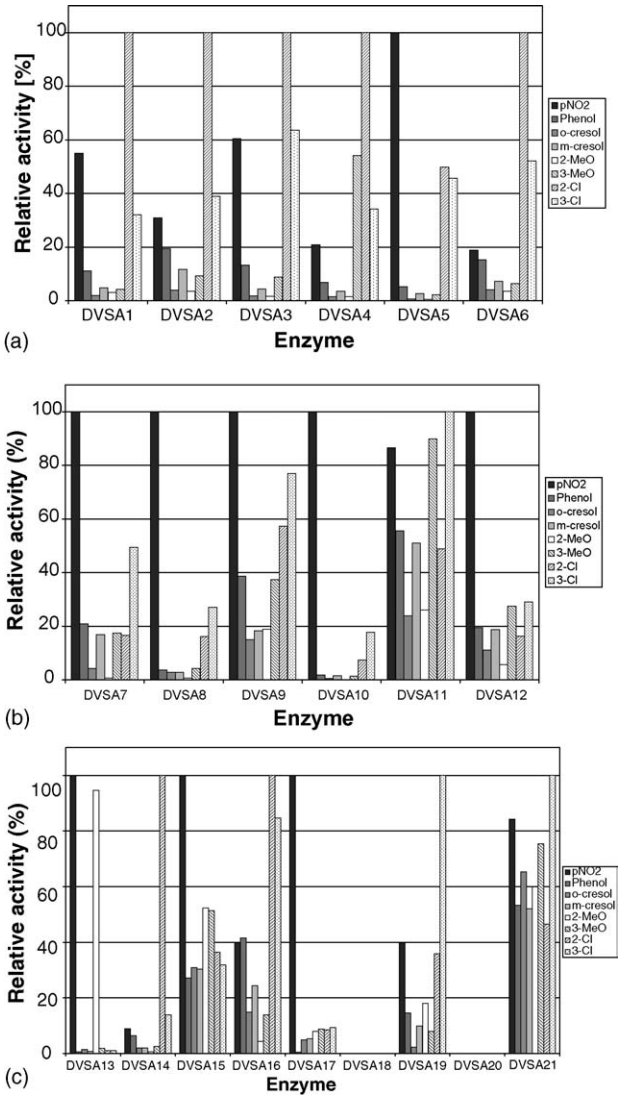


Fig. 2. (a)–(c) Relative activity for the Diversa enzymes. Experimental conditions for the assay are detailed in Section 2. Specific activities ranged from 0 to 3.9 U/mg. Enzymes DVSA18 and DVSA20 did not show any activity towards these substrates.

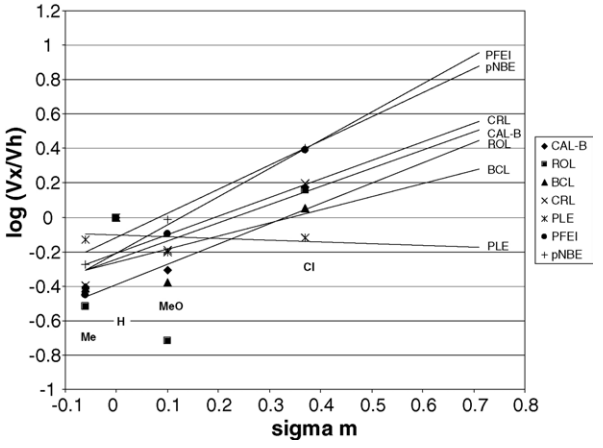


Fig. 3. Hammett plot for the cleavage of the *m*-substituted phenyl esters by seven lipases and esterases. Activity was measured as detailed in Section 2.

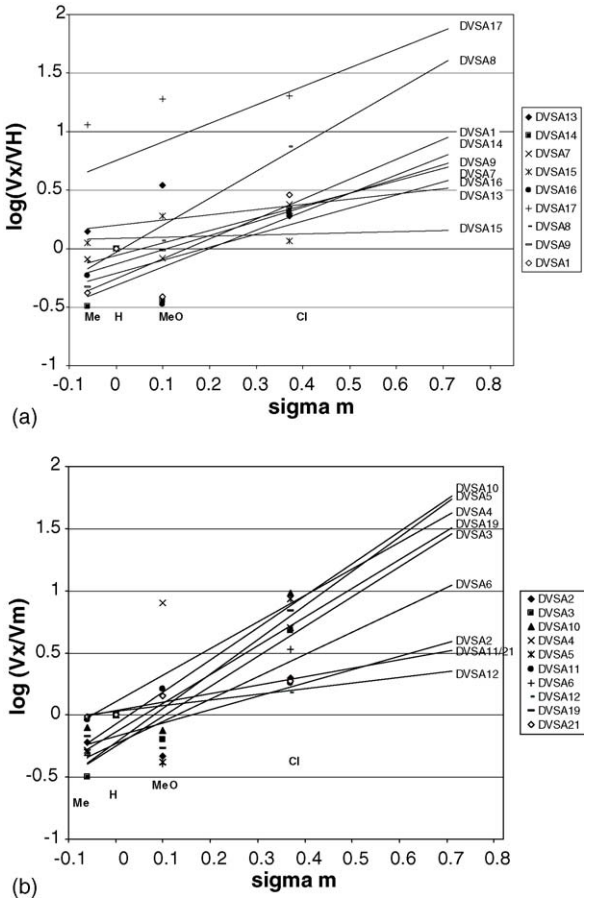


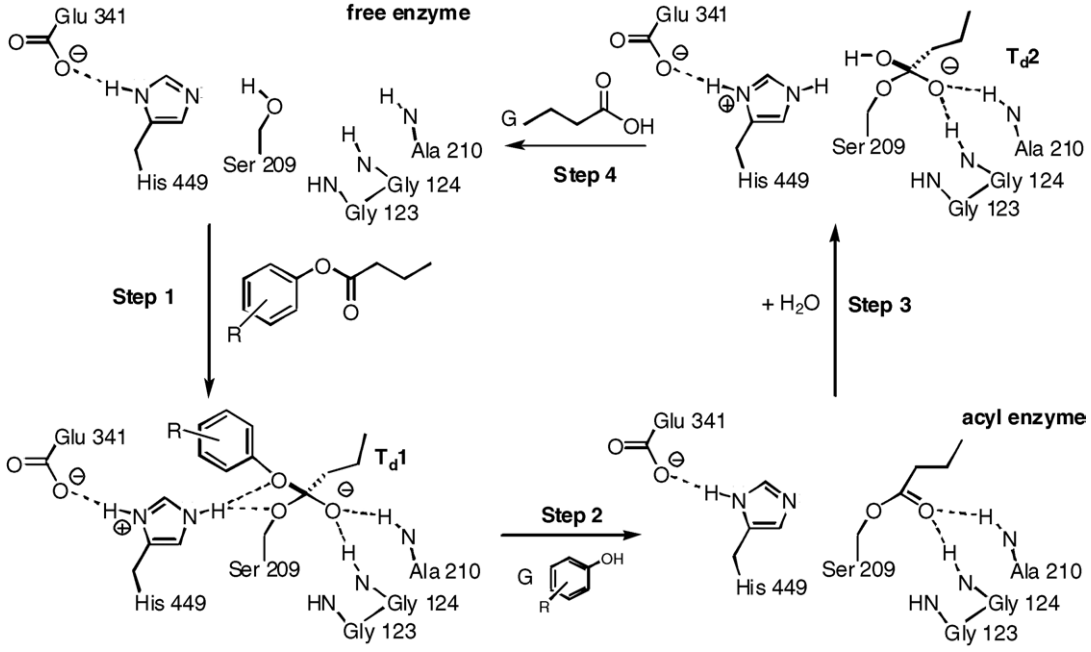
Fig. 4. (a)–(b) Hammett plot for the cleavage of the *m*-substituted phenyl esters by Diversa enzymes. Activity was measured as detailed in Section 2.

CRL). Step 1 involves the formation of an ester-enzyme tetrahedral intermediate, which is negatively charged. From this intermediate, the phenol is released (Step 2), yielding the acyl-enzyme. The acyl enzyme is then hydrolyzed (Step 3) through a second tetrahedral intermediate to finally release the acid and free enzyme (Step 4).

The influence of the phenol substituent is important to the first two steps, formation and collapse of the first tetrahedral intermediate T_d1. The presence of electron withdrawing substituents would effectively stabilize the tetrahedral intermediate and thus facilitate Step 1 through inductive effects. Similarly these substituents will stabilize the phenol leaving group, facilitating Step 2.

For all enzymes, the phenol moiety has to be accommodated in the enzyme active site such that the scissile ester bond is appropriately presented to the enzyme catalytic machinery. Depending on the enzyme, this fit may be dependent on both electronic and steric parameters of the enzyme active site and the substrate. For some enzymes, the steric and electronic architecture of the active site may place little or no restriction on the leaving alcohol. In the absence of other effects, the rates of these enzymes would be expected to parallel the chemical reactivities of the substrates, as described above, and the behaviour of such enzymes could be theoretically predicted. While this appears to be the case for some enzymes tested here, others clearly deviate from this predicted trend. In these cases, additional factors influencing interactions between the enzyme and the phenol moiety have to be considered.

Although this study is not predictive to guide the selection of appropriate lipase/esterase for the hydrolysis of different



Scheme 3. Mechanism of lipase/esterase-catalyzed hydrolysis of the phenol butanoates. The amino acid numbering corresponds to the active site of lipase from *C. rugosa*, CRL [2].

fatty acid esters, it highlights that the rate of hydrolysis of these substrates can be very sensitive to the structure of the leaving alcohol. This is an important consideration for the design of high throughput esterase and lipase assays.

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