Insights from molecular dynamics simulations into pH-dependent enantioselective hydrolysis of ibuprofen esters by *Candida rugosa* lipase

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An interesting observation was found during our continued studies on the hydrolysis of ibuprofen esters by *Candida rugosa* lipase (CRL). An important role is played by pH in the stereospecific hydrolysis of these esters. The flap region of CRL plays a significant role in the access of the substrate to the active site of the enzyme. At pH 5.6, 48% of the methyl ester and 5% of the butyl ester of ibuprofen were hydrolysed in 5.5 h, whereas at pH 7.2, 9% of methyl ester and 45% of the butyl ester of ibuprofen was hydrolysed in an identical reaction time using CRL. This lead us to assume that CRL prefers the methyl ester of ibuprofen as a substrate at an acidic pH and the butyl ester of ibuprofen at a neutral pH. Therefore, in order to understand the role of pH in the substrate selection by CRL for the esters of ibuprofen we used the crystallographic coordinates of the open form of the CRL (1CRL) for molecular dynamics (MD) simulations under acidic and neutral conditions for 2 ns using GROMACS. The final structures obtained after simulation in acidic and neutral conditions were compared with the energy-minimized structure, and the root-mean-square deviations (r.m.s.d.s) were calculated. The r.m.s.d. of the CRL flap at neutral pH was found to be greater than that of the CRL flap at acidic pH. The extent to which the flap opens at neutral pH allowed the bulkier substrate, the butyl ester of ibuprofen, to diffuse into the active site and provides the best enzyme–substrate fit for this specific substrate. At acidic pH there is a decreased opening of the flap thereby accommodating a more compact substrate, namely the methyl ester of ibuprofen. Thus, simulation experiments using MD provide reasonable insight for the pH-dependent substrate selectivity of CRL in aqueous environments.

**Keywords:** *Candida rugosa* lipase/enantioselectivity/ibuprofen esters/molecular dynamics simulations/non-steroidal anti-inflammatory drug

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Introduction

Triglycerol lipases (EC 3.1.1.3) are enzymes that hydrolyse triglycerides to free fatty acids and glycerol. One characteristic feature of the reactivity of lipases is that they react rapidly with insoluble rather than with soluble ester substrates in aqueous solutions. Lipases are today used as chiral catalysts in organic synthesis (Bornscheuer and Kazlauskas, 1999).

Lipases have also been useful as an industrial catalyst for the resolution of racemic acids and alcohols and in a variety of fields such as household detergents (Falch, 1991), oils and fats (Macrae, 1983), dairy (Izco *et al.*, 2000), organic media (Cardenas *et al.*, 2001), leather and paper industries.

Ibuprofen is a non-steroidal anti-inflammatory drug and is generally administered as a racemate (Hutt and Caldwell, 1984). The inversion of the R(−) to S(+) that occurs for aryl-propionic acids may have both toxicological and therapeutic implications when the drug is administered as a racemate (Adam *et al.*, 1976; Beck *et al.*, 1991). Resolution of racemic ibuprofen by *Candida rugosa* lipase (CRL) can be achieved through stereospecific esterification in non-aqueous media (Mustranta, 1992), while in aqueous media this approach is extended to stereospecific hydrolysis of the corresponding esters.

The factors that trigger the activation at the lipid–water interface have been discussed. It was suggested that a conformational change in the enzyme might lead to the increased activity (Sarda and Desnuelle, 1958). Using standard protein structure determination methods, it was found that there was a minimal structural change in lipases except at the flap region (comparison of open and closed forms with and without inhibitor state that explicitly). Maximum conformational change observed during activation involves a rigid body hinge type motion of a single helix (lid/flap) (Hernosos *et al.*, 1997).

The structure of CRL (Grochulski *et al.*, 1993) revealed that the active site Ser209, His449 and Glu341 is covered by a flap/lid, e.g. *Bacillus subtilis*. A variety of triglycerol lipase structures have been solved (Brandy *et al.*, 1990; Winkler *et al.*, 1990) and not all lipases have their active site shielded by a flap/lid, e.g. *Bacillus subtilis* (Poudroyen *et al.*, 2001) lipase lacks the flap. Recently, the sequencing of *Cephaloleia presignis* (Espinosa *et al.*, 2000).
lipase revealed that there was a very different initial sequence (first 28 amino acids from the N terminal) with respect to the many known lipases.

All the enzymes in water have a pH-activity profile. In porcine pancreatic lipase, it is important to have the enzyme in a buffer with the optimal pH for activity prior to dehydration for use in organic solvents with low or no buffering capacity (Zaks and Klibanov, 1985). The effect of pH in the water phase localized around the enzyme in organic solvents using hydrophobic esters of fluorescein as indicators (Brown et al., 1990; Valivety et al., 1990) has been studied. There have been very few reports concerning the effect of pH on the enantioselectivity of enzymes (Schnider et al., 1984). Studies have concluded that pH can greatly influence the activity and the enantioselectivity of lipase, which might probably result from the conformational changes of lipase with pH variation. It was observed that the best enantioselectivity of CRL towards 2-chloroethyl ether of ketoprofen was obtained at pH 2.2 with the enzyme still active and stable (Liu et al., 1999). The activity of human gastric lipase (HGL) with short chain and long chain triacylglycerols was determined as a function of pH (Gargouri et al., 1986). The maximum specific activity was reached at pH 6.0 with tributyrin with long chain triacylglycerols and the maximum lipolytic activity was obtained in the pH range of 4.5–5.5. The influence of pH on the specific activities of rabbit gastric lipase (RGL) (Moreau et al., 1988) and dog gastric lipase (DGL) (Carrière et al., 1991) was also tested using short chain, medium chain and long chain triacylglycerols as substrates. The specific activity of DGL on long chain triacylglycerol (TAG) reached a maximum at pH 4.0, whereas it was 6.0 and 5.5 on short chain TAG with HGL and RGL. The medium chains were hydrolysed at an optimum pH of 6.0.

Molecular dynamics (MD) simulations have been used in analysing the conformations the solute acquired in various solvent conditions (Peters and Bywater, 1999). The residues in the flap play a major part in the activity of Thermomyces lanuginose lipase (Cajal et al., 2000). Previous MD simulations performed on Rhizomucor miehei lipase (Peters et al., 1999a,b; Peters and Bywater, 1999) provided considerable insight into the lid dynamics and the possible manner in which the protein may accommodate incoming substrates. MD simulation studies have also been used to elucidate the effect of point mutation in Humicola lanuginosa (Peters et al., 1998).

In this paper, we describe an interesting phenomenon that was observed as we were trying to enhance the yield of (S+) ibuprofen by the hydrolysis of its esters by CRL. We found that on varying the pH of the reaction mixture there was a differential selectivity of the enzyme for the substrate at two different pH conditions. We used MD simulations to study this differential substrate selection by CRL under varying conditions of pH and attributed it to the differential motion of the flap using MD simulations.

| Table I. The percentage conversion of esters of ibuprofen at 5.5 h at pH 5.6 and 7.2 |
|-----------------|--------|--------|
| pH              | 5.6    | 7.2    |
| Time taken (h)  | 5.5    | 5.5    |
| Butyl ester (%) | 5      | 48     |
| Methyl ester (%)| 48     | 9      |

Figure 1. CRL after 2000 ps simulation superimposed with its energy-minimized conformation. The blue and red flaps represent the energy-minimized and simulated conformations, respectively. The pH states were modelled by protonating all the lysines and histidines were protonated at ND1 (neutral state) and NE2 (acidic state) site.

Materials and methods

CRL (EC 3.1.1.3) with specific activity of 835 U/mg assayed using olive oil as substrate was purchased from Sigma Chemical Company (St Louis, MO, USA). The methyl, ethyl, propyl and butyl esters of ibuprofen were synthesized using standard racemic ibuprofen and the corresponding primary alcohols. 1-Hexane, acetone and isopropyl alcohol were of HPLC grade and were purchased from SD Fine Chemicals Co., Bombay, India. Trifluoroacetic acid (TFA) was purchased from Fluka Chemical Company, Switzerland. Citric acid, KH₂PO₄ and K₂HPO₄ of analytical grade were obtained from SD Fine Chemicals Co., Na₂H₂PO₄ (Merck) and Na₂HPO₄ (Qualigens Fine Chemicals), used in the preparation of sodium phosphate buffer pH 7.2, were of analytical grade. The (S+) form of ibuprofen was purchased from Aldrich Company and was also used as the standard. A water bath shaker, pH meter (Elico model no. L1-120), high performance liquid chromatograph (HPLC, Shimadzu LC6A) with PDA detector, reverse phase C18 column and Chiral Diacel OD (Shibata et al., 1989) column were used for the present study. The detection wavelength was set to 254 nm. MD simulations were performed in a cluster, running GROMACS (Lindahl et al.,...)
The esters of (+)-ibuprofen were prepared using (+)-ibuprofen.

**Hydrolysis reaction**

**Methyl ester of ibuprofen.** Unless otherwise stated, the reaction mixture consisted of 10 mg of CRL dispersed in 10 ml of citric acid phosphate buffer (McIlvaine buffer pH 5.6) and 20 ml of racemic ibuprofen ester. The mixture was agitated at 170 r.p.m. at 37°C for 5.5 h. A known aliquot of the reaction mixture was vortexed with twice the volume of n-hexane. Ibuprofen and ibuprofen ester, being immiscible in water and soluble in hexane, were extracted into the organic phase and 10 ml of the organic phase was analysed by HPLC.

**1-Butyl ester of ibuprofen.** Sodium phosphate buffer pH 7.2 was prepared and 50 mg of CRL was added to 10 ml of the buffer. The reaction was started by adding 1 mM of the substrate butyl ester of ibuprofen (262 mg). The mixture was kept at 37°C in a water bath shaker at 150 r.p.m. for 48 h.

**Chiral analysis of S(+) ibuprofen**

**Methyl ester of ibuprofen.** The extract was treated with sodium bicarbonate (0.5 M) solution to convert the acid (ibuprofen) to its sodium salt that later gets extracted in the aqueous phase, while the unreacted ester remained in the organic phase. The aqueous phase was treated with 3 N HCl to regenerate ibuprofen and the acidic solution was shaken with 1-hexane to extract the regenerated ibuprofen in the organic phase. The yield of S(+) ibuprofen was monitored by HPLC using a Chiral Diacel OD column capable of separating the R(−) and S(+) enantiomers. The mobile phase was hexane–2-propanol–TFA (100:1:0.1), the column temperature was 30°C and the flow rate was 1 ml/min.

**1-Butyl ester of ibuprofen.** The reaction mixture was extracted in two 5 ml portions of HPLC grade hexane. The organic layer was collected and to this 10 ml of 0.5 ml sodium bicarbonate (NaHCO₃) was added and shaken well in a separating funnel. The aqueous layer was collected and acidified using 3 N HCl. The acidified NaHCO₃ was re-extracted in 10 ml of hexane using a separating funnel. The aqueous layer was discarded and the organic fraction was dried over anhydrous magnesium sulphate (pre-dried in an oven at 100°C overnight). The hexane was totally evaporated in a rotary evaporator; to this 1 ml of hexane was added, vortexed and used for the chiral analysis in HPLC. The enantiomer of ibuprofen was identified by analysing the samples in HPLC using a chiral column and comparing with the standard resolved enantiomer of ibuprofen. The mobile phase consisted of hexane–isopropanol–TFA in the ratio of 100:1:0.1. The flow rate was set to 1 ml/min and the peaks were detected at 254 nm.

**Molecular dynamics simulation**

As a starting point for the simulations the coordinates of the open form of CRL (1CRL.pdb) was obtained from the Protein Data Bank (www.rcsb.org/pdb). The MD simulation was performed using GROMACS. The leap-frog algorithm for integrating Newton equations and the periodic boundary condition was applied. All bonds were constrained using LINCS (Hess et al., 1997) and 15600 water molecules were added to 5126 and 5124 solute atoms and the box size was 8.77900×8.62200×7.27500 nm, at the end there were 51 929 and 51 927 atoms to be simulated in the acidic and neutral
environments, respectively. During energy minimization the steepest descents algorithm was used and in both pH cases the minima was reached in 159 steps. MD was performed with a time step of 2 fs and the coordinates were saved every 1000 steps. The potential energy was conserved during the MD. The MD simulations were performed at 300 K and Berendsen temperature coupling (Berendsen et al., 1984) was applied. MOLMOL (Koradi et al., 1996) was used to calculate the root-mean-square deviation (r.m.s.d.) of the flap of CRL in the acidic and neutral conditions to the energy-minimized structure.

Results

**Enzymes require appropriate protonation of catalytic residues for optimal activity**

Huge-Jensen et al. (1987) reported that the hydrolysis of olive oil catalysed by commercial lipase is sensitive to the nature of buffer pH owing to an enzyme–buffer interaction. The desired amount of active site protonation can be achieved by altering the pH of the reaction mixture. Hence, pH of the reaction mixture can play an important role in optimal enzyme activity. Generally, it is a well known fact that enzymes lose their activity under extreme pH conditions. Gu et al. (1986) have shown that the enzymatic resolution of methyl ester of aryl propionic acids by CRL was slow at pH 8.0.

We have studied the enzymatic hydrolysis of methyl and butyl esters of ibuprofen at pH 5.6 and pH 7.2 using CRL. The two ester substrates differ in the nature of the alkyl group of the alcohol moiety. Interestingly, we found that CRL prefers the methyl ester of ibuprofen at pH 5.6 and the butyl ester of ibuprofen at pH 7.2 (Table I). The reactions proved to be highly enantioselective with only the $S(\text{+})$ ibuprofen esters getting hydrolysed. Table I shows the yield of the methyl and butyl esters of $S(\text{+})$ ibuprofen at different pH conditions taken as a measure of the rate of transformation. The observed (%) yield

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**Figure 3.** The dynamic picture of CRL simulated over 2 ns in an acidic environment superposed on to its energy-minimized conformation. The black and grey flaps represent simulated and energy-minimized conformations.
of $S(+)$ ibuprofen in the CRL catalysed hydrolysis was found to decrease with increase in the chain length of the alkyl group at pH 5.6. The reverse is observed at pH 7.2 with preference being accorded to the butyl ester of $S(+)$ ibuprofen.

It was observed that the hydrolysis of methyl ester at pH 7.2 and butyl ester at pH 5.6 was not largely preferred. This property of CRL to differentially select substrates in different pH conditions is intriguing as to why CRL prefers butyl over methyl ester at neutral pH and vice versa at acidic pH. Clearly the reaction is controlled by the nature of the substrates and pH of the reaction mixtures.

In order to understand the molecular basis of this phenomenon, we performed MD simulation of the open form of the CRL in different pH conditions using GROMACS force field. On analysis of the amino acid sequence of CRL, we identified all the titrable amino acid groups in the enzyme and compared their $pK_a$ values (Voet and Voet, 1995). Amino acids glutamic acid and aspartic acid have $pK_a$ values <5.0 and hence it is not right to protonate them for simulation at pH 5.6 nor at pH 7.2. There are five histidines in the amino acid sequence of CRL which can be protonated/deprotonated as a function of pH. The amino acid lysine has a $pK_a$ value of >10 and therefore all solvent-exposed lysines in the enzyme are heavily protonated at pH 7.2 and pH 5.6.

The $pK_a$ value of the lysines and other titrable residues was calculated using Delphi (Honig and Nicholls, 1995). It showed that the percentage protonation of lysines was 99 and 96% at pH 5.6 and 7.2, respectively. Hence, we proceeded to model CRL with the assumption that all the lysines are protonated immaterial of which pH state the enzyme is in (pH 5.6 or 7.2). To incorporate a pH gradient only the histidines were protonated at NE2 and ND1 sites to model pH 5.6 and 7.2.

After 2 ns of simulation at pH 5.6 and 7.2 the flap of CRL showed a r.m.s.d. of 3.048 Å, as shown in Figure 1a and b, to the energy-minimized conformation. The black and grey flaps represent simulated and energy-minimized conformations.

![Figure 4. The snapshot picture of CRL simulated over 2 ns in a neutral environment superposed on to its energy-minimized conformation. The black and grey flaps represent simulated and energy-minimized conformations.](image-url)
This necessitated us to look into the possibility of lysines located in hydrophobic pockets or deep inside the enzyme because protonation of solvent-exposed lysines is highly feasible than those buried inside the protein or in hydrophobic pockets.

**Discussion**

Nielsen et al. (2001) have shown that lysines located in hydrophobic pockets experience a perturbed pKа. We identified three lysine residues in CRL whose solvent accessibility was low, located at positions 75, 180 and 404 (Table II) using the WHATIF program (Vriend, 1990). Thus, residues buried inside the enzyme have less chance for protonation due to the hydrophobic property of the surrounding residues (Barabas et al., 1997). However, these buried lysines will be protonated at lower pH than physiological pH (Nielsen et al., 2001).

Of the three buried lysines, K75 is surrounded by solvent-accessible residues and the K75 was assumed not to be in a hydrophobic pocket, thereby having negligible differential effect at the two different pH conditions. To further substantiate this, the closed form of CRL showed high solvent accessibility to K75 but K180 and K404 were inaccessible to the solvent at both the open and closed forms of CRL. We have thus attempted to study the role of lysines K180 and K404 located at the hydrophilic patch in understanding the conformation of the CRL flap at the two different pH states used in the study, and hence to explain the substrate specificity of CRL towards the butyl and methyl esters of ibuprofen.

The two buried lysines (K180 and K404) are fully protonated at pH 5.6 as compared with pH 7.2, and thus exist with a perturbed pKа in their micro-environment. This was exploited in the modelling of the pH states. The buried lysines at positions 180 and 404 were protonated along with all the other lysines to simulate the behaviour of CRL at pH 5.6 (shown in Figure 2). These two residues were left unprotonated during simulation at pH 7.2 with all the other lysines protonated. The protonation states for histidines were assigned by the MD program (GROMACS) which computes the hydrogen-bonding pattern of the protein, and indicates if the proton should be on the NE2 rather than the ND1 site or at both ND1 and NE2 sites of histidine.

The protonation states of histidines were as follows: His151 at ND1, His218 at ND1, His368 at ND1, His425 at NE2 and His 449 at ND1 and NE2. The structure of CRL was monitored over the 2000 ps of simulation time at the two pH conditions, as shown in Figures 3a–d and 4a–d.

The whole enzyme simulations were analysed by plotting the r.m.s.d., radius of gyration (RG) and the hydrogen bonds as a function of simulation time. The r.m.s.d. of the acidic (Figure 5a) and neutral states are very similar to each other. After 1600 ps the neutral system starts to equilibrate. The r.m.s.d. of the neutral system is a shade more than the acidic system. A comparison of the r.m.s.d. (Figure 5a), RG (Figure 5b) and hydrogen bond (Figure 5c) profiles of both the systems shows the micro-environment of the enzyme during the hydrolysis of esters of ibuprofen. We have given the system the initial 500 ps to equilibrate and have not included this time period in our analysis.

The flap in the acidic system moving towards a closed form and the flap in the neutral system moving to a more open conformation compared with the energy-minimized structure is shown in Figure 6. On analysing the r.m.s.d. of the flap we found that in the acidic environment the flap had moved by 6.09 Å towards the closed conformation, whereas in the neutral environment the flap opened further by 3.672 Å (calculated using MOLMOL) with respect to the energy-minimized structure.

Comparing the RG of the acidic and neutral systems (Figure 5b), we see the RG of the acidic system marginally increasing at 1000 ps and then dropping away, but the RG of the neutral system shows a dip at ~600 ps and surpasses the acidic system to rise to a peak value of 2.25 nm and dips at 1950 ps. The comparative analysis of the RG of the two systems reveals that the acidic system shows a greater gyration and peaks at 1000 ps and the hydrogen profile (Figure 5c) for.
the same time shows an increase in the number of hydrogen bonds in the acidic system, pointing to a more compact enzyme at that time. Apart from this the hydrogen bond profiles of the enzyme in acidic and neutral environments were very much similar.

Conclusion

Protein structures determined by standard structure determining techniques do not give the conformation of the protein in the lowest energy state that it would have in solution. MD simulation will, given enough time, find the lowest energy conformation in the region of phase space surrounding that of the starting structure. While crystal structures are minimum energy structures in the crystal, this may not represent the minimum energy in solution.

From the simulation of CRL in a hydrophilic environment at two different pH states we see that the CRL flap moves towards the closed conformation in an acidic environment. This results in a decreased availability of the hydrophobic zone produced by the lesser extent of the opening up of the flap. We deduce that the conformation of the enzyme in an acidic environment is more favourable for smaller and less hydrophobic side chain substrates. In neutral environment, simulations show that the flap opens ‘more’ as compared with an acidic environment.

The further opening of the flap produces a larger hydrophobic zone on CRL favourable for larger substrates with a more hydrophobic side chain to gain access to the active site. This leads us to extrapolate that the choice of substrate is very much dependent on pH for CRL. The motion of the flap is necessary for the substrate entering the active site.

Researchers worldwide are trying to understand the structure–function relationship of lipases, especially CRL, and we hope to shed more light on the significant role played by pH. The theoretical studies done here by means of MD simulations have successfully elucidated why a more hydrophobic butyl ester of ibuprofen is preferred by CRL at a neutral pH and the methyl ester of ibuprofen at an acidic pH. This pH-based structure–function relationship helps contribute to a better understanding of the functionality of CRL by probing the flap dynamics.

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