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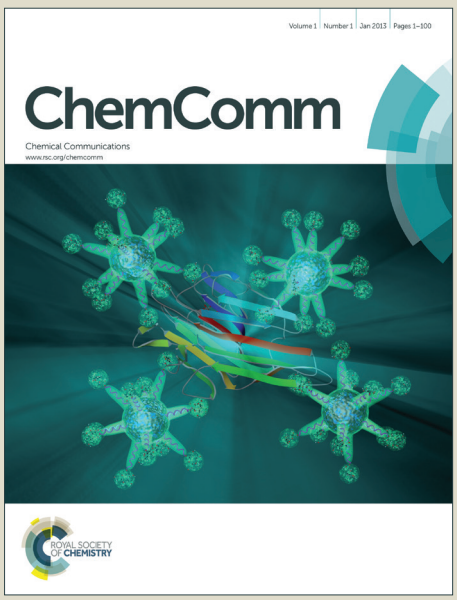

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Saturation mutagenesis in selected amino acids to shift *Pseudomonas* sp. acidic lipase Lip I.3 substrate specificity and activity

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Abstract. Several *Pseudomonas* sp CR611 Lip I.3 mutants with overall increased activity and a shift towards longer chain substrates were constructed. Substitution of residues Y29 and W310 by smaller amino acids provided increased activity on C_{18}-substrates. Residues G152 and S154, modified to study their influence on interfacial activation, displayed a five and eleven fold increased activity.

Lipases (EC 3.1.1.-) are among the most used biocatalysts in biotechnology[1-3] and constitute the most important group of enzymes for synthetic organic chemistry[4, 5] or biodiesel production[6-8]. However, there is still need for new enzymes to fulfil the requirements of different industrial processes. Therefore, there is substantial interest in developing new or improved lipases for use in food, biomedical or chemical industries[5, 9].

The study of biodiversity is a valuable tool for the discovery of new lypolitic enzymes useful for biocatalysis[10-12]. In a previous work[13] we reported the characterization of a subfamily I.3 acidic lipase –Lip I.3− from *Pseudomonas* sp. CR-611[12]. Biocatalytic applications of subfamily I.3 lipases are scarcely known, with only two crystallized lipases: SML from *Serratia marcescens*[14], and PML from *Pseudomonas* sp. MIS38[15, 16]. Despite a high sequence similarity, subfamily I.3 lipases display differences in their catalytic behaviour[13, 17]. In fact, SML structure is very similar to that of PML[14, 16] but as Lip I.3, it does not show interfacial activation. These facts show the necessity of investigating the influence of small sequence/structure variations to understand the catalytic behaviour of each lipase, and prompted us to get a greater understanding of subfamily I.3 lipases through the modification of Lip I.3.

Subfamily I.3 lipases show preference for medium chain substrates[13, 16, 18], a feature that is not favourable for certain biotechnological applications, like biodiesel production. Different techniques can be used to modify lipase activity. Immobilization has proved a powerful strategy to improve lipase activity towards a particular substrate[19, 20]. Directed evolution and rational protein design techniques[21-25] may allow to adjust the enzyme properties to the needs of industrial processes through identification of specific residues that can be mutated in order to improve a specific property such as substrate preference[22, 26, 27]. However, no modifications of subfamily I.3 lipases have been attempted before. In this work we used a semi-rational mutagenesis approach to modify the catalytic properties of Lip I.3 and to determine the influence of minor changes in the catalytic behaviour of this lipase, by comparison with PML and SML. Lip I.3 mutants with improved activity on medium and long chain substrates were obtained, contributing to a better understanding of the relationship between structure and activity of subfamily I.3 lipases.

Previous attempts to produce Lip I.3 in soluble form in *E. coli* were unsuccessful[13]; thus, a new expression system based on *P. aeruginosa* PAO1 Δ*lipH*[28] was set up for high throughput screening of Lip I.3 activity. *P. aeruginosa* PAO1 Δ*lipH* is a mutant strain deficient on the specific foldase LipH required for proper folding of its lipases[29], thus showing no lypolitic activity. Presence of fully active Lip I.3 in culture supernatants was confirmed by comparing the activity of strain PAO1 Δ*lipH* (pBBR1MCS-LipI.3) with that of strain PAO1 Δ*lipH* (pBBR1MCS), used as a negative control (Figure 1). For selection of mutation sites, a 3D model structure of Lip I.3 was constructed based on reported structures of *Pseudomonas* sp. MIS38 lipase (PML) in closed[16] and open[15] conformation. From the model obtained, several amino acids were selected for either saturation or site directed mutagenesis.

Three bulky residues –Y^{29}, F^{166} and W^{310}–, located at the substrate entrance tunnel, were selected as targets for saturation mutagenesis. N^{344} was also selected for saturation mutagenesis based on a shift found in *Candida antarctica* lipase LIP4 towards short chain compounds when mutation V344Q was introduced[30]. Asparagine (N) of Lip I.3 at position 344, structurally similar to the glutamine (Q) of mutated LIP4 could account for its preference for short chain substrates. Accordingly, we performed an NNK library to change N^{344} by other residues which might enhance activity towards long chain substrates. Activity on 4-methylumbelliferyl(MUF)-heptanoate and MUF-oleate was measured for approximately 100 clones from each library (positions 29, 166, 310 and 344) in order to have a complete coverage of all possible mutations with 95% confidence. Caster informatics tool was used to analyze the influence of each position on activity[31].

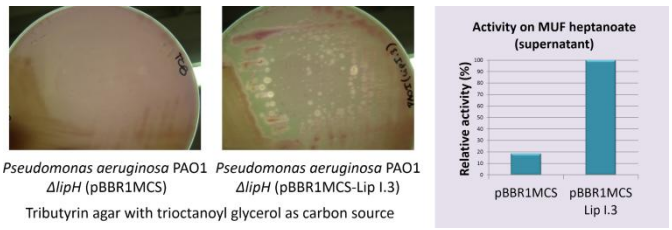

Figure 1: Construction of *P. aeruginosa* PAO1 Δ*lipH* (pBBR1MCS-LipI.3) yielded a suitable strain for production of extracellular and active Lip I.3.

An activity increase of 40 % was observed for mutants in libraries Y29X and W310X when compared to wild type Lip I.3, assayed on the same substrate (Table 1). For positions Y29 and W310, an average 4.5 and 2.5 percentage, respectively, of mutants bearing over 130% activity with respect to wild type Lip I.3 were found, whereas the percentage of those showing activity below 50% was 3.6 and 3.3, respectively (Table 1), suggesting that those are critical positions for activity. Saturation mutagenesis at position F166 produced 1% mutations bearing activity over 130% of that of wild type Lip I.3 whereas unexpectedly, changes at asparagine 344[30] produced no mutants with sufficient activity to be considered as improved Lip I.3 variants (Table 1). Mutations Y29C, Y29T, W310M, W310F and W310Q were found among the mutants showing better activities on MUF-oleate (Table 2).

Table 1: Analysis of the hydrolytic activity on MUF-oleate of different Lip I.3 variants obtained after saturation mutagenesis for residues Y29, F166, W310 and N344. Relative activity was calculated considering wild type Lip I.3 activity as 100 %.

	Saturation mutation			
	Y29X	**F166X**	**W310X**	**N344X**
Total studied variants	112	96	120	112
Relative activity of best mutant	137	131	142	124
Relative activity ≥ 130 % (% of total studied variants)	4.5	1.0	2.5	0.0
Relative activity < 50 % (% of total studied variants)	3.6	2.1	3.3	0.9

Moreover, mutants Y29C and Y29T displayed the same increase of activity when assayed on MUF-heptanoate, while mutants W310M and W310F retained Lip I.3 wild type activity on this substrate. These results are in agreement with the fact that modifications on substrate specificity are more frequent when mutations occur near the active site[32] and residues Y29 and W310 are closer to the active site than the other modified amino acids. Moreover, removal of bulky residues contributes indeed to widen up the substrate entrance tunnel, thus allowing acceptance of a bigger substrate as MUF-oleate. Variants Y29C and Y29T are the best candidates for biocatalytic processes using long chain substrates like biodiesel production[6, 8, 33, 34], or medium chain substrates like in tertiary or secondary alcohol enantioresolution[35-37]. A 3D modelling confirmed that mutations at position Y29 bring to an important opening of the catalytic site (Figure 2).

Table 2: Relative activity on MUF-oleate of the best mutant variants of Lip I.3 obtained by saturation mutagenesis.

Mutation	Relative activity (%)[a]
W310M	142
Y29C	137
W310F	137
Y29T	136
W310Q	133

[a] Relative activity = 100×Mutant activity/ Lip I.3 wt activity

When tyrosine 29, a bulky and aromatic amino acid, is substituted by a threonine (Figure 2b) or a cysteine (Figure 2c), both smaller residues, the distance between them and the nucleophile serine is almost double compared to that found on wild type Lip I.3 (Figure 2a), leaving more space for longer substrates entrance, without hindering the possibility of using shorter substrates. For substitutions at position W310, even though the distance increase between modified residues at position W310 and the nucleophile serine is smaller (Figure 2d,e,f), good activities on MUF-oleate were obtained due to a wider space generation. Instead, lack of activity increase on MUF-heptanoate could be explained for these mutants by a distortion of the enzyme structure caused by a charge effect due to the lower hydrophobicity of the replacing amino acids (Met, Phe or Gln) compared to that of wild type Trp, which would make the catalytic site more accessible to larger than to shorter substrates, as previously suggested[38].

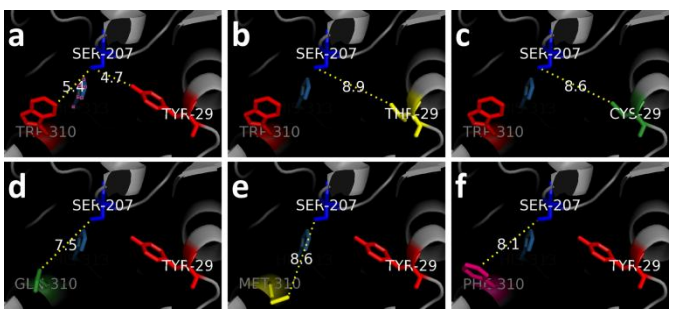

Figure 2: 3D computational modelling of mutants Y29C (b), Y29T (c), W310Q (d), W310M (e) and W310F (f) compared to wild type Lip I.3 (a). The model shows the influence of the mutations on widening the substrate entrance channel.

An alignment of LipI.3, PML and SML sequences revealed some differences in Lid1 sequence (Supplementary Material Figure S1). Residues 149, 152, 154 and 165 presented variations among

the three lipases, in a context of a highly conserved sequence. Since interfacial activation is a major difference among the three enzymes, we decided to study those variable residues in Lid 1. The mutations chosen were I149N, I149S, G152L, I149N/G152L, S154T and A165G. Hydrolytic activity of all generated variants was measured on MUF-heptanoate and MUF-oleate, and their kinetic behaviour was studied (Figure 3). Although these mutations are located on Lid 1, our kinetic studies showed that none of the variants displayed interfacial activation (Figure 3a), indicating that other residues apart from those located in Lid 1 are essential for interfacial activation. Best activity results in terms of catalytic efficiency were obtained for mutants G152L and S154T (Figure 3b). S154T showed a 5-fold activity increase on MUF-heptanoate, while G152L was twice more active on this substrate than the wild type enzyme. This gain in activity is probably related with an alteration of the closed to open conformation equilibrium of Lip I.3. In the case of lipases, immobilization of the enzyme[20], as well as addition of detergent[13], has been shown to influence enzyme activity by favouring the open conformation of the enzyme. Thus, differences on enzyme aggregation among the different mutants can also be associated to the observed change in activity. Although we cannot rule out this possibility, an analysis of the modelled enzyme was made in order to provide a rational explanation for those changes. In variant G152L, the glycine found in Lip I.3 and SML was changed by a lysine, like in PML[39]. Analysis of mutated Lip I.3 model structure (Figure 4) revealed that the new lysine side chain at position 152 might cause steric impediments, affecting residues of Lid 2 in the closed conformation, thus favouring the open conformation of the enzyme, a fact that would then favour its catalytic effectiveness. Interestingly, the double mutant I149N/G152L showed the same activity on MUF-heptanoate as that of wild type Lip I.3, indicating that mutation at residue 149 is detrimental to the improvement associated with the G152L substitution.

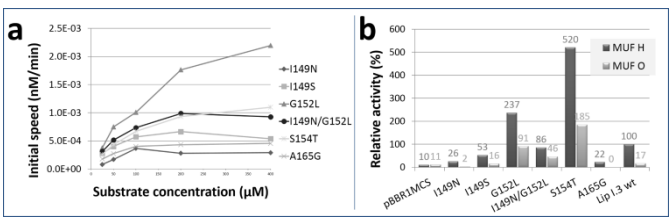

Figure 3: (a) kinetic studies and (**b)** relative activity of site directed mutagenesis mutants compared to wild type Lip I.3.

Increase of activity of variants G152L and S154T on MUF-oleate was even more dramatic, rising to an 11-fold increase for mutant S154T. This activity increase was even higher than that of mutants at the substrate entrance tunnel (see above), suggesting that other residues apart from those of the active site entrance tunnel are involved in substrate recognition and binding. The high activity increase shown by mutant S154T could be due to a wider opening of Lid 1 plus an additional hydrophobicity gain resulting from substitution of the OH group from S154 by a methyl group provided by the replacing T (Supplementary Material Figure S3). Moreover, when Lip I.3 was modelled, a calcium ion coordinated in Ca1 position was found in both PML[15] and SML[14], which was not present in the model of Lip I.3. Absence of such calcium in Lip I.3 is associated to the presence of serine instead of threonine at position 118, thus missing the coordination site for Ca1. Modelling PML T118S variant showed that this single change was enough to cause loss of Ca1 in the model. As residue 154 is very close to amino acid 118 in the open conformation, mutation S154T might be reversing the effect of the difference at position 118, helping to coordinate a calcium ion at position Ca1, like in PML[15]. This would contribute to restore an anchoring site for Lid 1, favouring the open conformation and boosting activity. A crystal structure of the mutant and wild type Lip I.3 will be necessary in order to confirm this hypothesis.

The increased activity associated with some of the mutated residues demonstrates that modifications in the structure of Lid 1 can clearly help to gain increased catalytic activity and to widen up the range of substrate acceptance. In fact, mutations on the lid have already been reported to influence substrate specificity or other biochemical properties of lipases[40-42]. Regarding substrate chain length preference, mutations on the lid of *Pseudomonas fragi* lipase (PFL) shifted substrate specificity towards longer acyl chain substrates[41]; however, lid swapping in *C. rugosa* lipases did not contribute to the acceptance of larger acyl chain fatty acids[40]. Dynamic modelling and crystallization of wild type Lip I.3 and the mutants obtained may better enlighten the causes of the acquired improvement in activity.

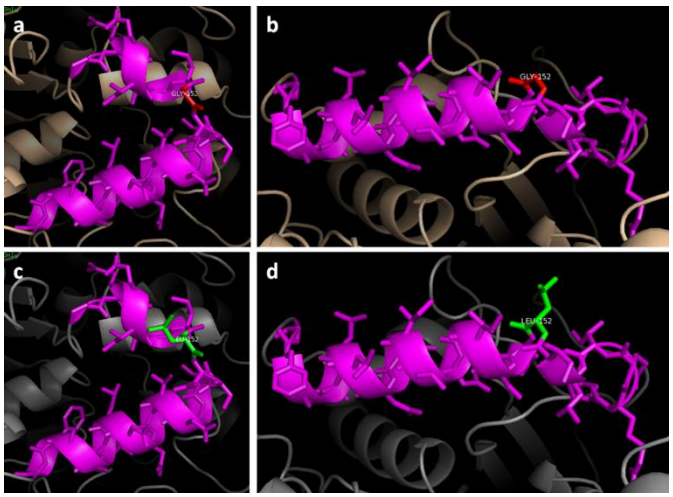

Figure 4: Lid 1 in the closed (a,c) and open (b,d) conformations of Lip I.3 wild type (a,b) and Lip I.3 variant G152**L** (c,d). Residue G152 is shown in red, L152 appears in green and the rest of amino acids of Lid 1 are depicted in purple.

Conclusions

A semirational design strategy was applied to expand Lip I.3 substrate specificity towards long chain fatty acid substrates in order to improve its potential for biotechnological applications such as biodiesel production. Saturation mutagenesis of bulky residues (Y29 and W310) in the substrate entrance tunnel proved a useful strategy, since substitution by smaller amino acids at those positions produced mutants with 40 % increased activity towards MUF-oleate. Site directed mutagenesis performed on Lid 1 generated an interesting variant (S154T) showing a 5 and 11-fold increased activity towards MUF-heptanoate and MUF-oleate, respectively. Modelling studies indicate that threonine at position 154 could produce a gain in hydrophobicity and serve as a coordination site for Ca1, thus providing an anchoring site for Lid 1 in the open conformation and explaining the large increase in activity associated with such a subtle modification. Results obtained by altering Lid 1 residues indicate that the Lid constitutes a hot spot for

mutations oriented to shift substrate specificity. Although no other studies have previously been done for improvement of subfamily I.3 enzymes, the results obtained here are in agreement with those reported by Santarossa for PFL lipase, a *Pseudomonas* subfamily I.1 enzyme. The coincident results among these studies, suggest that lipase Lids can be a general target for altering lipase substrate specificity in *Pseudomonas* lipases.

Notes and references:

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Electronic Supplementary Material available.

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