Tailoring recombinant lipases: keeping the His-tag favors esterification reactions, removing it favors hydrolysis reactions

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We determined the effect of the His-tag on the structure, activity, stability and immobilization of LipC12, a highly active lipase from a metagenomic library. We purified LipC12 with a N-terminal His-tag and then removed the tag using tobacco etch virus (TEV) protease. Circular dichroism analysis showed that the overall structure of LipC12 was largely unaffected by His-tag removal. The specific hydrolytic activities against natural and artificial substrates were significantly increased by the removal of the His-tag. On the other hand, His-tagged LipC12 was significantly more active and stable in the presence of polar organic solvents than untagged LipC12. The immobilization efficiency on Immobead 150 was 100% for both forms of LipC12 and protein desorption studies confirmed that the His-tag does not participate in the covalent binding of the enzyme. In the case of immobilized LipC12, the His-tag negatively influenced the hydrolytic activity, as it had for the free lipase, however, it positively influenced the esterification activity. These results raise the possibility of tailoring recombinant lipases for different applications, where the His-tag may be retained or removed, as appropriate for the desired activity.

Affinity tags have gained importance over the last decades as a purification tool for recombinant proteins1. Such tags include arg-tags, the calmodulin-binding peptide, cellulose-binding domains, DsbA, the c-myc-tag, glutathione S-transferase, the FLAG-tag, the HAT-tag, the maltose-binding protein, NusA, the S-tag, the SBP-tag, the Steep-tag, thioredoxin and His-tags2. Of these, His-tags (i.e. the polyhistidine affinity tag) are the most used, with approximately 25% of all protein structures in the Protein Data Bank (PDB) having been obtained for His-tagged proteins3. His-tagged proteins are purified by metal affinity chromatography, since the imidazole ring of the histidine establishes coordination bonds with immobilized ions (such as Co2+, Ni2+, Cu2+ or Zn2+)2. His-tags can be used for purifying proteins, under either non-denaturing or denaturing conditions, and the recombinant proteins can be eluted under mild conditions4,5. In addition to simplifying purification, His-tags are designed to optimize protein expression and therefore result in high yields. They also allow the detection of the recombinant protein6,7.

His-tags are bulky and might affect the conformation of the enzyme. The polyhistidine region typically consists of six consecutive histidine residues, but it can vary from two to ten histidine residues. In addition, His-tagged heterologous proteins that are expressed in Escherichia coli using the pET vector series8 contain several other amino acid residues. For example, use of the vector pET28a leads to the addition of a total of 20 residues at the N-terminal, of which only six are histidine residues.

His-tags are often used in the expression of recombinant lipases. Lipases are of great industrial interest due to their diverse biotechnological applications. They are efficient catalysts not only in conventional aqueous media, but also in non-conventional media, such as organic solvents, ionic liquids, supercritical fluids, and eutectic solvents9,10. Depending on the medium, they catalyze reactions of hydrolysis, esterification, transesterification, acetylation and lactonization11.

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Despite the potential that His-tags have to affect conformation and activity, to date, few studies have assessed their influence on the properties of lipases. In one study, the His-tagged *Staphylococcus xylosus* lipase (His-SXL), overexpressed in *E. coli*, had altered kinetic parameters, altered stereo- and regioselectivity and altered thermostability, compared to the native lipase (SXL)\(^1\)\(^2\)\(^3\). In another study, a recombinant *S. aureus* lipase (SAL), expressed in *E. coli* with a His-tag at the N-terminal, had a lower specific activity for the hydrolysis of tributyrin than the native lipase\(^4\).

In our laboratory, LipC12, a recombinant lipase containing a His-tag at the N-terminal, was originally isolated from a metagenomic library and characterized by Glogauer *et al.*\(^5\). Later, LipC12 was immobilized on Immobead 150. One immobilization preparation was obtained using the crude extract obtained after expression of the enzyme and cell breakage, while another was obtained using a preparation that had been purified from the crude extract using a nickel affinity column\(^6\)\(^7\)\(^8\). During these immobilization studies, Madalozzo *et al.*\(^9\) suggested that the presence of the His-tag might contribute to selective immobilization of His-LipC12 from the crude extract onto Immobead 150. Their argument was that, at the pH of 7.5 used for the immobilization, the histidine residues in the tag would be mostly deprotonated (the pKa of the histidine side chains in the His-tag is reported as 6.0)\(^10\) and this would favor nucleophilic attack by these side chains on the oxirane group of the support. However, they did not undertake studies to confirm that the His-tag was actually involved in immobilization.

In this work, we systematically investigated the effects of the His-tag on the structure, activity, stability and immobilization of LipC12. We used two strategies for the expression and removal of the His-tag from LipC12: (1) the lipC12 gene was cloned into pET28a for the expression of the His-tag on the N-terminal of the enzyme\(^11\)\(^12\)\(^13\) and this homolog was compared with the other recombinant form of LipC12, which was produced by cloning the lipC12 gene into pET29a for the expression of recombinant LipC12 (denominated rLipC12) without the His-tag; (2) the lipC12 gene was cloned into pTEV5 for the expression of the His-tag at the N-terminal of the enzyme, with the inclusion of a tobacco etch virus (TEV) cleavage site, enabling posterior His-tag removal\(^14\).

**Results**

All results in this section are presented as the mean of triplicate determinations \(\pm\) standard deviation of the mean.

**Properties of LipC12 expressed with and without His-tag.** Although previous reports have suggested that the His-tag negatively affects the hydrolytic activity of lipases\(^12\)\(^13\), it is not a consensus. In this work, we compared the activities and the immobilization behavior of LipC12 expressed with and without the His-tag. In order to do so, we prepared a crude soluble fraction containing LipC12 with His-tag and CSF-rLipC12 (crude soluble fraction containing LipC12 without His-tag), respectively, through overexpression in *E. coli* BL21(DE3). In each case, the cell pellet was disrupted by sonication, the crude extract was then centrifuged, and the crude soluble fraction, containing either His-LipC12 or rLipC12, was used for activity and immobilization tests.

The specific hydrolytic activities of these crude soluble fractions were determined in a pHStat against olive oil. The crude soluble fraction containing rLipC12 had a significantly higher specific activity (1938 \(\pm\) 82 U mg\(^{-1}\)) than the His-LipC12 fraction (1469 \(\pm\) 63 U mg\(^{-1}\)) \((P < 0.05)\). This difference in specific hydrolytic activity was not due to differences in protein expression: densitometry analysis of SDS-PAGE gels of the two crude soluble fractions showed that the lipases contributed similar percentages of the overall protein. In the case of His-LipC12, the lipase band (at ~33 kDa) contributed 18.6% \(\pm\) 0.8% of the overall protein, while in the case of rLipC12, the lipase band (at 31 kDa) contributed 18.0% \(\pm\) 0.3% (Supplementary Fig. S2).

Subsequently, we investigated the suggestion that the covalent immobilization of CSF-His-LipC12 on the commercial support Immobead 150 involves the His-tag\(^15\). We used previously established conditions\(^14\) (i.e. a loading of 200 mg of protein per gram of support) for immobilization of CSF-His-LipC12 and CSF-rLipC12. The kinetics of immobilization were followed by monitoring the residual olive-oil-hydrolyzing activity of the supernatant. The profiles were very similar (Fig. 1), with immobilization efficiencies of 80% being obtained at 8 h for both preparations (Supplementary Table S1). Hereafter, the immobilized preparations are referred to as I-CSF-His-LipC12 and I-CSF-rLipC12.

After immobilization, the olive-oil-hydrolyzing activities of the two LipC12 preparations were determined in the pHStat. As had occurred with the soluble preparations of LipC12, I-CSF-rLipC12 had a significantly higher hydrolytic activity (421 \(\pm\) 24 U g\(^{-1}\) support) than I-His-LipC12 (303 \(\pm\) 21 U g\(^{-1}\)) \((P < 0.05)\).

Both immobilized preparations were then used to esterify oleic acid with ethanol in \(n\)-hexane. The esterification activities for both preparations were not significantly different, 66 \(\pm\) 0 U g\(^{-1}\) for I-CSF-rLipC12 and 61 \(\pm\) 1 U g\(^{-1}\) for I-CSF-His-LipC12 \((P > 0.05)\).

Together, these results suggest that the His-tag decreases the hydrolytic activity of both the free and immobilized LipC12. Further studies using purified untagged and His-tagged LipC12 were needed to confirm these results, they are reported in the next subsection.

**Properties of purified LipC12 with and without cleavable His\(_{TEV}\)-Tag.** We cloned lipC12 into the vector pTEV5, which introduces the TEV protease cleavage site between the His-tag and the target protein. The resulting LipC12 variant, denominated His\(_{TEV}\)-LipC12, was overexpressed in *E. coli* BL21(DE3) and purified using immobilized metal affinity chromatography. The His-tag was later cleaved by TEV protease, with 100% efficiency, as confirmed by western blot (Fig. 2). The cleaved variant was denominated cLipC12. This strategy allowed the cost-effective production of both His\(_{TEV}\)-LipC12 and untagged cLipC12, with high purity being obtained in a one-step affinity process (Supplementary Fig. S1).

The lipC12 gene encodes 293 amino acids and rLipC12 has a theoretical molecular weight of 31.2 kDa. His\(_{TEV}\)-LipC12 has 27 additional residues at its N-terminal (MSYYHHHHHHHDYDIPTSENLYFQGASH) and...
has a theoretical molecular weight of 34.5 kDa. After TEV protease cleavage, four of these additional residues (GASH) remain at the N-terminal, such that cLipC12 has a theoretical molecular weight of 31.6 kDa. These theoretical molecular weights were confirmed by SDS-PAGE (Supplementary Fig. 3S).

Several authors have used TEV protease to cleave the His-tag from different target proteins. The amount of TEV required is usually empirically determined, with ratios of target protein to TEV between 100:1 and 5:1 being tested\(^20\). Our results showed complete cleavage under mild conditions (8 °C for 20 h) using a ratio of 10:1.

**Structural analysis of HiSTEV5-LipC12 and cLipC12 by circular dichroism.** The far-UV CD spectra of both HiSTEV5-LipC12 and cLipC12 showed typical negative absorption signals of α-helices at about 208 nm and 222 nm, as well as negative absorption signals of antiparallel β-sheets at 215 nm (Fig. 3A). Positive absorption peaks at about 190 nm were not seen because the dynode voltage was above 600 V, resulting in unreliable data\(^21\). This CD profile is similar to those shown by other lipases\(^22-25\). The CD spectra for HiSTEV5-LipC12 and cLipC12 overlap completely in the far-UV (250-200 nm) and near-UV (350-250 nm) regions, indicating that the His-tag does not induce structural rearrangements in LipC12. The CD spectra of HiSTEV5-LipC12 and LipC12 were further analyzed by K2D3 online software; for each protein, the estimate was an α-helix content of 36% and a β-sheet content of 20%. There is no previous study in which CD spectra have been used to characterize the structural changes of lipases due to the presence of a His-tag, but CD spectra have been used to show that a His-tag induces structural changes in zinc finger protein ZNF191 (243–368)\(^26\).

We also compared the thermal stabilities of HiSTEV5-LipC12 and LipC12 by recording CD data at 222 nm to determine the melting temperature (Tm) (Fig. 3B). The Tm decreased by only 1 °C in the presence of the His-tag, which suggests the His-tag has a minimum impact on the thermal stability of LipC12.
Hydrolytic activity of HisTEVS-LipC12 and cLipC12.

The specific olive-oil-hydrolyzing activities were 2555 ± 17 U mg⁻¹ for HisTEVS-LipC12 and 4590 ± 101 U mg⁻¹ for cLipC12. The significantly lower hydrolytic activity in the presence of the His-tag (P < 0.05) confirms the results obtained above, where the hydrolytic activity of CSF-His-LipC12 was lower than that of CSF-rLipC12 and the hydrolytic activity of I-CSF-His-LipC12 was lower than that of I-CSF-rLipC12 (see the first subsection of the results). We also evaluated the activities of HisTEVS-LipC12 and cLipC12 in the hydrolysis of natural substrates with a range of acyl chain lengths. For all substrates, the activity of HisTEVS-LipC12 was significantly lower than that of cLipC12 (P < 0.05) (Table 1).

Hydrolytic activity of HisTEVS-LipC12 and cLipC12 against pNPD.

Under the standard reaction conditions (see methods section) for the hydrolysis of p-nitrophenyl decanoate (pNPD), cLipC12 had a specific activity of 325 ± 20 U mg⁻¹, which is 1.7-fold higher than that of HisTEVS-LipC12 (192 ± 5 U mg⁻¹).

The effect of the pNPD concentration on the initial hydrolysis rate was determined for both HisTEVS-LipC12 and cLipC12 under the standard reaction conditions (Supplementary Fig. S4). The Michaelis-Menten equation was fitted to the data obtained with both enzymes using non-linear regression. In both cases, the coefficient of determination was greater than 0.99. Table 2 shows the parameter values that gave the best fits.

Hydrolytic activity of HisTEVS-LipC12 and cLipC12 against different triacylglycerols.

| Substrate     | Relative hydrolytic activities |
|---------------|---------------------------------|
|               | HisTEVS-LipC12 | cLipC12 |
| Olive oil     | 100 ± 4         | 173 ± 4 |
| Triolein (C18)| 109 ± 5         | 170 ± 6 |
| Tricaprylin (C8)| 188 ± 2       | 345 ± 9 |
| Tributyryl (C4)| 304 ± 8       | 611 ± 10|

Table 1. Relative hydrolytic activities of HisTEVS-LipC12 and cLipC12 against different triacylglycerols. Measurements were performed by the titration method using an automatic titrator pHStat. Values represent the mean of triplicates ± standard deviations of the mean.

| Enzyme          | $K_M$ (mM) | $k_{cat}$ (min⁻¹) | $k_{cat}/K_M$ (mM⁻¹ min⁻¹) |
|-----------------|------------|-------------------|-----------------------------|
| HisTEVS-LipC12  | 1.77 (1.56–2.02) | 152 (143–162) | 86 (80–92) |
| cLipC12         | 1.33 (1.17–1.51) | 151 (144–160) | 114 (105–123) |

Table 2. Apparent kinetic constants for p-nitrophenyl decanoate hydrolysis of HisTEVS-LipC12 and LipC12. The values between parentheses are the 95% confidence intervals.

Activity and stability in organic solvents of HisTEVS-LipC12 and cLipC12.

The use of organic solvents instead of water in biocatalytic processes involving lipases has intensified over the last few decades. Lipases must therefore have both high activity and high stability in organic solvents if they are to be useful biocatalytic tools. Based on the difference in activity found in aqueous media using untagged LipC12 (CSF-rLipC12,
cLipC12 and I-CSF-rLipC12) and His-tagged LipC12 (His-LipC12, HisTEVS-LipC12 and I-His-LipC12), we investigated HisTEVS-LipC12 and cLipC12 with respect to their pNPD-hydrolyzing activity and stability in various concentrations of different polar organic solvents (ethanol, isopropanol, methanol, propanol, acetone, acetonitrile, DMF and DMSO). The activities were expressed as relative activities, in other words, as percentages of the corresponding activity in 50 mM Tris-HCl buffer (pH 7.5) used in the standard reaction.

HisTEVS-LipC12 showed significantly higher activity in the presence of acetone, DMF and DMSO than cLipC12. For both variants of LipC12, the relative activities were lower than 50% for all organic solvents and at all concentrations tested, except for DMSO (Fig. 4A). For this solvent, concentrations up to 40% activated both enzymes, with HisTEVS-LipC12 being activated to a greater extent than cLipC12. It is well known that polar organic solvents like methanol, ethanol, propanol and butanol inhibit lipase activity. On the other hand, DMSO has been shown to activate lipases several-fold. Although the mechanism of this activation has not been elucidated, it has been suggested that DMSO stabilizes the transition state by replacing water in the enzyme-substrate complex.

To evaluate the stability in organic solvents, HisTEVS-LipC12 and cLipC12 were incubated for 3 h at 25 °C in two concentrations (20% and 40% (v/v)) of the polar organic solvents (Fig. 4B). The hydrolytic activity against pNPD was then determined under standard conditions (using final enzyme concentrations of 7 nM for HisTEVS-LipC12 and 5 nM for cLipC12). In most cases, HisTEVS-LipC12 was more stable than cLipC12 (Fig. 4B). However, in 20% ethanol, 40% methanol, 40% acetone, 40% acetonitrile and 40% DMF, the residual activities of the two enzymes were not significantly different (P > 0.05). The activity of HisTEVS-LipC12 increased approximately 1.2-fold after incubation in 20% methanol, 20% acetonitrile and 20% DMF. The stability of HisTEVS-LipC12 in DMSO was not higher than in the other hydrophilic solvents, since its residual activity was 80% after incubation in 40% DMSO (Fig. 4B). Both HisTEVS-LipC12 and cLipC12 were more stable in polar organic solvents than lipase P21 of Pseudomonas fluorescens, which had residual activities below 50% after incubation for 2 h at 30 °C in 3:1 (v/v) solutions of methanol, ethanol, acetone, isopropanol and butanol in water.

**Immobilization of HisTEVS-LipC12 and cLipC12.** Purified His-LipC12 was immobilized on Immobead 150 in previous work. The authors proposed that the His-tag was involved in the covalent bonding with the support. To investigate this question, we immobilized HisTEVS-LipC12 and cLipC12 onto the same support.

For both HisTEVS-LipC12 and cLipC12, an immobilization efficiency of 100% was obtained using a protein loading of 10 mg g⁻¹. Despite this high immobilization efficiency, the retention of hydrolytic activity in aqueous medium, measured using triolein, was low (0.21% for HisTEVS-LipC12 and 0.27% for cLipC12). A possible
than that of I-cLipC12, which gave conversions at 9 h below 68%.

To verify the stability of I-His TEVS -LipC12 organic solvents (Fig. 4B).

The flexible His-tag may interfere with the opening of the lid or it might interact with the active site of other variants without the His-tag (cLipC12) was higher than that of the variant with the His-tag (His TEVS -LipC12) at both above and below the critical micellar concentration (CMC), which is 0.24 mM for Triton X-100 and 0.08 mM for Nonidet P-40. The effects of the detergents on the two lipases were similar, although the activity of the LipC12 variant without the His-tag (cLipC12) was higher than that of I-HisTEVS -LipC12 (54 ± 1 U g⁻¹). On the other hand, the presence of the His-tag increased esterification activity for the substrates octanoic acid, lauric acid, palmitic acid and oleic acid (Table 3). At longer reaction times, the performance of I-HisTEVS -LipC12, with conversions at 9 h above 90%, was also higher than that of I-cLipC12, which gave conversions at 9 h below 68%. To verify the stability of I-HisTEVS -LipC12 and I-cLipC12 throughout the reaction, the immobilized preparations were recovered after 10 h by filtration, washed with n-hexane, dried, and the residual hydrolytic activities against olive oil were measured. The losses of olive-oil-hydrolyzing activity were about 50% for I-HisTEVS -LipC12 and 74% for I-cLipC12, showing that the removal of the His-tag decreases the stability of I-cLipC12 in the reaction medium, which initially contains ethanol and n-hexane, with ethyl caprylate, ethyl laurate, ethyl palmitate or ethyl oleate being produced during the reactions. This result is in agreement with the lower stability found for cLipC12 when it was incubated in polar organic solvents (Fig. 4B).

### Discussion

Our work represents the first time that a His-tagged lipase (I-His TEVS -LipC12) has been shown to have lower hydrolytic activity but higher esterification activity, when compared to a similar recombinant enzyme without the His-tag (I-cLipC12). Since lipases are used both for hydrolysis and synthesis reactions, this opens up the possibility of removing the His-tag or not, after expression, in order to tailor the enzyme for the specific application that is intended.

Various previous studies have reported the effects of affinity tags on hydrolytic and esterification activities, as we discuss below. In our comparisons with the literature, we will use the prefix “wt” to indicate a wild type lipase, indicating that the lipase was not produced using molecular biology techniques. The prefix “r” will be used to indicate a recombinant lipase without a His-tag. Since His-tag proteins are recombinant proteins, where possible, we will compare the His-tagged lipases with similar recombinant lipases. Where the authors did not give results for a recombinant lipase, we will undertake comparisons with the wild type lipase.

The most important result of our work is that a His-tagged lipase (I-His TEVS -LipC12) has been shown to have lower hydrolytic activity but promoted esterification activity. His-tagged variants of LipC12 consistently gave lower hydrolytic activities in aqueous media. Both free (CSF-His-LipC12 and His TEVS -LipC12) and immobilized (I-CSF-His-LipC12 and I-His TEVS -LipC12) enzymes had lower specific olive-oil-hydrolyzing activities than their non-His-tagged counterparts in free (CSF-rLipC12 and cLipC12) and immobilized (I-CSF-rLipC12 and I-cLipC12) forms. Likewise, for the hydrolysis of pNPD, the value of $k_{cat}/K_M$ for His TEVS -LipC12 was 75% of the value obtained for cLipC12. The situation was different for esterification of oleic acid with ethanol in n-hexane. In this case, I-CSF-His-LipC12 and I-CSF-rLipC12 had similar activities, but, on the other hand, I-HisTEVS -LipC12 had both a significantly higher activity and a better stability in the reaction medium than did I-cLipC12.

We suggest that these results might be explained as follows: the His-tag is highly flexible in aqueous medium and some of its movements may temporarily block access of substrates to the active site. There are two possibilities: the flexible His-tag may interfere with the opening of the lid or it might interact with the active site of other lipases in the reaction medium, as has been shown by Majoreck et al. To investigate the second possibility, we added two non-ionic detergents (Triton X-100 and Nonidet P-40) to reaction media during the hydrolysis of pNPD, with the aim of separating aggregates of enzyme molecules. For both detergents, we used concentrations both above and below the critical micellar concentration (CMC), which is 0.24 mM for Triton X-100 and 0.08 mM for Nonidet P-40. The effects of the detergents on the two lipases were similar, although the activity of the LipC12 variant without the His-tag (cLipC12) was higher than that of the variant with the His-tag (His TEVS -LipC12) at all detergent concentrations (Supplementary Figure S7). This result suggests that there is no interaction between different lipase molecules and, therefore, that the second possibility does not occur.

### Table 3. Esterification activity of I-cLipC12 and I-His TEVS -LipC12 with fatty acids of different chain lengths and ethanol as the alcohol.

| Fatty acid       | I-cLipC12 Activity (U g⁻¹) | Conversion (%)¹ | I-His TEVS -LipC12 Activity (U g⁻¹) | Conversion (%)¹ |
|------------------|--------------------------|-----------------|-------------------------------------|-----------------|
| Octanoic acid (C8:0) | 1.51 ± 0.03              | 26.1 ± 0.5      | 5.90 ± 0.03                         | 90.8 ± 1.7      |
| Lauric acid (C12:0)     | 1.65 ± 0.08              | 26.4 ± 2.5      | 6.84 ± 0.15                         | 100.0 ± 0.7     |
| Palmitic acid (C16:0)    | 2.32 ± 0.19              | 42.2 ± 2.2      | 7.88 ± 0.22                         | 97.5 ± 0.2      |
| Oleic acid (C18:1)      | 5.26 ± 0.03              | 67.9 ± 0.3      | 9.21 ± 0.01                         | 96.8 ± 0.4      |

¹Conversion of free fatty acids at 9 h of the esterification reaction. Values represent the mean of triplicates ± standard deviation of the mean.

To confirm covalent immobilization, these immobilized preparations were boiled for 30 min in a solution containing SDS. After SDS-PAGE, in both cases, approximately 8% of the immobilized protein desorbed. This shows that, for both immobilized enzymes, most of the protein was covalently attached to Immobead 150. Since both variants produced the same results, it can be deduced that the His-tag does not favor the covalent binding of LipC12 to Immobead 150.

In accordance with the findings for the variants of LipC12 that had been immobilized from the crude soluble fraction (I-CSF-His-LipC12 and I-CSF-rLipC12), the olive-oil-hydrolyzing activity of I-cLipC12 (123 ± 2 U g⁻¹) was higher than that of I-His TEVS -LipC12 (54 ± 1 U g⁻¹). On the other hand, the presence of the His-tag increased esterification activity for the substrates octanoic acid, lauric acid, palmitic acid and oleic acid (Table 3). At longer reaction times, the performance of I-His TEVS -LipC12, with conversions at 9 h above 90%, was also higher than that of I-cLipC12, which gave conversions at 9 h below 68%. To verify the stability of I-His TEVS -LipC12 and I-cLipC12 throughout the reaction, the immobilized preparations were recovered after 10 h by filtration, washed with n-hexane, dried, and the residual hydrolytic activities against olive oil were measured. The losses of olive-oil-hydrolyzing activity were about 50% for I-His TEVS -LipC12 and 74% for I-cLipC12, showing that the removal of the His-tag decreases the stability of I-cLipC12 in the reaction medium, which initially contains ethanol and n-hexane, with ethyl caprylate, ethyl laurate, ethyl palmitate or ethyl oleate being produced during the reactions. This result is in agreement with the lower stability found for cLipC12 when it was incubated in polar organic solvents (Fig. 4B).
On the other hand, proteins tend to be less flexible in hydrophobic organic solvents of relatively high log P values, such as n-hexane\(^2\), which we used in the esterification reactions. In this medium, the His-tag would also be less flexible, for example, it might be trapped within the solvation layer, and therefore unable to interfere with the access of substrates to the active site. NMR analysis or molecular dynamics studies of LipC12, with and without the His-tag in aqueous solution and in the presence of organic solvents, would be needed to confirm these suggestions. We recognize that our explanation does not explain why I-His\textsuperscript{TEVS}-LipC12 had a significantly higher esterification activity than I-cLipC12. However, His-tags can have subtle effects on protein structure\(^3\). For example, although a His-tag did not affect the overall structure of myoglobin, it did alter the picosecond-scale dynamic of the protein; this change can affect the recognition of ligands\(^4\).

The effects of His-tags on lipases have been most studied with respect to their effects on hydrolytic activities (Table 4). Various authors have compared specific activities of lipases with and without His-tags, using several different substrates. Results are variable, with His-tags having been reported to have no effect or to cause decreases and increases of specific hydrolytic activity. In our case, the His-tag decreased the specific olive-oil-hydrolyzing activities (values were 2555 U mg\(^{-1}\) for His\textsuperscript{TEVS}-LipC12 and 4590 U mg\(^{-1}\) for cLipC12). Decreases in specific hydrolytic activity due to the His-tag have been reported previously. For example, for \textit{Staphylococcus aureus} lipase 3 (SAL3), the specific activity of His-SAL3 against tributyrin (4000 U mg\(^{-1}\)) was lower than that of the recombinant untagged enzyme (r-SAL3, 4500 U mg\(^{-1}\))\(^4\). Likewise, for the hydrolysis of tributyrin (TC4) and olive oil by the lipase of \textit{S. xylosus}, His-SXL had lower specific activities (1500 and 850 U mg\(^{-1}\), respectively, for TC4 and olive oil)\(^5\) than did r-SXL (2000 and 1400 U mg\(^{-1}\), respectively, for TC4 and olive oil)\(^5\). However, in some cases, the specific hydrolytic activity was higher with the His-tag. For example, in the case of SAL3, the specific activity against olive oil of His-SAL3 (1300 U mg\(^{-1}\)) was higher than that of r-SAL3 (1100 U mg\(^{-1}\)). Similar results were obtained for the hydrolysis of TC4 and olive oil by the lipase of \textit{S. simulans} (SXL)\(^6\). The specific activities of His-SLAL against both these substrates (980 and 940 U mg\(^{-1}\), respectively, for TC4 and olive oil) were higher than those of r-SLAL (850 U mg\(^{-1}\), for both TC4 and olive oil)\(^7\). In some cases, the His-tag caused no significant difference in specific hydrolytic activities. This has been reported for the hydrolysis of pNPB, pNPL and carboxylfluorescein diacetate by Lipase B of \textit{Candida antarctica} (CALB)\(^8\), for the hydrolysis of olive oil and TC4 by turkey pancreatic lipase (TPL)\(^9\) and for the hydrolysis of TC4, trioctanoin (TC8) and olive oil by the lipase of \textit{Fusarium solani} (Table 4).

Some authors have analyzed the effect of the His-tag on kinetic parameters (Table 4). Again, the results are quite variable. In our case, the His-tag did not have any effect on \(k_{\text{cat}}\) for hydrolysis of \(p\)-nitrophenyl decanoate, but increased \(K_M\) 1.3-fold, such that \(k_{\text{cat}}/K_M\) for His\textsuperscript{TEVS}-LipC12 was 75% of the value obtained for cLipC12. In other words, the presence of the His-tag decreases the catalytic efficiency of LipC12 with \(p\)NPD as the substrate. Decreases in \(k_{\text{cat}}/K_M\) with the presence of the His-tag have been reported previously for: SAL3 with TC4 as the substrate, \(k_{\text{cat}}/K_M\) for His-SAL3 was 74% of the value obtained with r-SAL3\(^3\); for SXL with TC8 as the substrate, \(k_{\text{cat}}/K_M\) was 80% of the value obtained for wt-SXL\(^12\) and for various variants of CALB with a fusion protein at the N-terminus and a His-tag at the C-terminus, the His-tagged proteins had values of \(k_{\text{cat}}/K_M\) for the hydrolysis of \(p\)-nitrophenyl butyrate (pNPB) that were from 85 to 98% of the value for the unmodified CALB\(^13\). Increases in \(k_{\text{cat}}/K_M\) have also been reported for His-SAL3 with TC8 as the substrate: for SAL3, the value of \(k_{\text{cat}}/K_M\) for His-SAL3 was 1.5-fold higher than that for r-SAL3\(^14\) while for SXL, the value of \(k_{\text{cat}}/K_M\) for His-SXL was 1.2-fold greater than that for wt-SXL\(^12\). There are also some reports of no significant differences in the kinetic parameters between the His-tagged and non-tagged forms: this was the case for a recombinant CALB, for both pNPB and \(p\)-nitrophenol laurate (pNPL) as substrates\(^9\) and for TPL with TC4 as the substrate\(^9\).

Significantly, our work represents the first report of a His-tag leading to an increase in esterification activity of a lipase: for the synthesis of ethyl-olate in n-hexane, the specific activity of I-His\textsuperscript{TEVS}-LipC12 (9.21 ± 0.03 U g\(^{-1}\)) was significantly higher than that of I-cLipC12 (5.26 ± 0.03 U g\(^{-1}\)). The only previous study of the effects of His-tags on esterification activity, undertaken by Horchani \textit{et al.}\(^14\), involved the synthesis of butyl oleate in n-hexane with three immobilized variants of \textit{S. aureus} lipase 3: I-wt-SAL3, I-r-SAL3 and I-His-SAL3. Based on the conversions at the first sampling time of that study, 4 h, one can infer that r-SAL3 and His-SAL3 gave similar initial esterification rates, which were much lower than that obtained with I-wt-SAL3. Horchani \textit{et al.}\(^14\) followed the reaction profiles over 24 h, with I-wt-SAL3 giving a conversion of around 70% in 20 h and both r-SAL3 and His-SAL3 giving much lower conversions at this time, of around 20%\(^14\). The key difference from our work is that, in the case of SAL3, the presence of the His-tag decreased stability in the solvents tested, although it should be noted that n-hexane is much less polar than the solvents that we used in our stability studies. The results of Horchani \textit{et al.}\(^14\) suggest, indirectly, that both I-r-SAL3 and I-His-SAL3 are denatured by polar solvents. The explanation is as follows. The low final conversions that they obtained at 20 h must have been caused by denaturation, as the reaction profiles had leveled out. Since both I-r-SAL3 and I-His-SAL3 had given residual activities above 80% after 24-h incubations in n-hexane in their stability study, the loss of activity of these preparations in the esterification reaction was not due to the presence of n-hexane. It is possible to infer, then, that both I-r-SAL3 and I-His-SAL3 were denatured by one of the reaction species involved in the synthesis of butyl oleate, possibly the butanol, with the presence of the His-tag not improving stability. Contrary to these results of Horchani \textit{et al.}\(^14\), in our case the presence of the His-tag did
Table 4. Effects of His-tags on lipases.

| Lipase                        | Tag                                | Aspect studied                                                                 | Key results for His-tagged protein relative to untagged protein | Article |
|-------------------------------|------------------------------------|-------------------------------------------------------------------------------|-----------------------------------------------------------------|---------|
| Metagenomic lipase LipC12, expressed in Escherichia coli | N-terminal His-tag (Six histidine residues) | Specific hydrolytic activities against TC4, TC8, TC18, olive oil, pNPD | Lower                                             | This work |
|                               |                                    | Hydrolytic activities against pNPD in presence of polar organic solvents          | Higher in acetone, DMF and DMSO                                      |         |
|                               |                                    | Stability in polar organic solvents                                             | Higher in methanol, acetonitrile and DMF                           |         |
|                               |                                    | Kinetic parameters for pNPD hydrolysis                                          | $K_{cat}$ higher and $k_{cat}/K_{M}$ lower for TC4, but not affected for TC8 | Moshah et al.12 |
|                               |                                    | Synthesis of ethyl oleate over 10 h with immobilized lipase                     | Higher                                                          |         |
| Staphylococcus xylosus Lipase (SXL) | N-terminal His-tag (Six histidine residues) | Kinetic parameters for hydrolysis of TC4 and TC8                              | $K_{cat}$ and $k_{cat}/K_{M}$ lower for TC4                          | Moshah et al.12 |
| Lipase B of Candida antarctica (CALB) expressed in Escherichia coli | N-terminal FLAG and C-terminal His-tag (Six histidine residues) | Specific hydrolytic activities against pNPB, pNP1 and CFDA | No difference                                                | Blank et al.38 |
|                               |                                    | Kinetic parameters for hydrolysis of pNPB and pNP1                              | No difference                                                      |         |
| Lipase B of Candida antarctica (CALB) | N-terminal His-tag (Six histidine residues) | Kinetic parameters for hydrolysis of pPNB                                      | $K_{cat}$, $k_{cat}$ and $k_{cat}/K_{M}$ lower for all tags            | Seo et al.51 |
| Staphylococcus aureus Lipase (SAL) | N-terminal His-tag (Six histidine residues) | Specific hydrolytic activities                                                 | Lower in tributyrin, higher in olive oil                           |         |
|                               |                                    | Kinetic parameters for hydrolysis of TC4                                       | $k_{cat}/K_{M}$ lower                                              |         |
|                               |                                    | Immobilization on CaCO₃                                                         | Immobilization efficiency lower                                   | Horchani et al.14 |
|                               |                                    | Synthesis of butyl oleate with immobilized lipase over 24 h                     | Lower conversion of oleic acid to ester                           |         |
|                               |                                    | Stability in organic solvents                                                  | Lower stability in organic solvents (n-hexane, tert-butanol and chloroform) for both free and immobilized forms |         |
| Lipases of Staphylococcus simulans (SSL & r-SL), S. xylosus (SXL & r-SXL) and S. aureus (SAL & r-SAL) | N-terminal His-tag (Six histidine residues) | Specific hydrolytic activities                                                 | Lower with TC4 and with olive oil for His-SXL, higher with TC4 and with olive oil for His-SLL | Horchani et al.57 |
| Turkey pancreatic lipase (TPL) | N-terminal His-tag (Six histidine residues) | Specific hydrolytic activities against olive oil and TC4                      | No significant difference                                          | Bou Ali et al.39 |
| Yarrowia lipolytica Lipase YLlip11 | N-terminal His-tag (Six histidine residues) | Specific hydrolytic activities against olive oil and TC4                      | No significant difference $k_{cat}$ and $k_{cat}/K_{M}$             | Jallouli et al.40 |
| Fusarium solani lipase expressed in Pichia pastoris | N-terminal His-tag (Six histidine residues) | Specific activities against TC4, TC8, olive oil, phospholipids (e.g. egg-yolk PC) and galactolipids (1,2-octanoyl-3-O-α-D-galactosyl-sn-glycerol (DiC8-MGDG)) | No difference                                                    | Kumari et al.40 |

Table 4. Effects of His-tags on lipases.

To improve stability within the esterification reaction medium, with the residual activity after 10 h being 54% for I-HisTEVS-LipC12 against only 24% for I-cLipC12.

In the case of immobilization of lipases, only one previous study has addressed the effect of His-tags. In this study, Horchani et al.14 immobilized wt-SAL3, r-SAL3 and His-SAL3 on CaCO₃. The immobilization yield was high for wt-SAL3 (79%). It was significantly lower for r-SAL3 (22%) and much lower still for His-SAL3 (2.5%), suggesting that the His-tag did interfere somehow with immobilization. This effect of the His-tag is different from the effect in the current work, in which the His-tag did not affect immobilization.

Our study demonstrates that the His-tag decreases the specific hydrolytic activities of both the free and immobilized forms of LipC12 against triacylglycerols. However, in several polar solvents, both the pNPD-hydrolyzing activity and the stability were favored by the presence of the His-tag. Importantly, our study represents the first time that a His-tag has increased esterification action. Our work gives insights into tailoring lipases for specific applications; for hydrolysis reactions, the His-tag should be removed from LipC12, while for esterification reactions, it should be retained.

**Methods**

**Bacterial strains, plasmids and protein expression.** LipC12 is a lipase isolated by Glogauer et al.15 from a metagenomic library prepared from DNA extracted from fat-contaminated soil of a waste treatment plant. The gene *lipC12* was cloned into pET28a(+) (this plasmid is referred to as pET28lipC12) to produce an N-terminal His-tagged protein (denominated His-LipC12) that has a theoretical molecular weight of 33.4 kDa. The *lipC12* gene was also cloned into pET29a(+). This was done by digesting pET28lipC12 with *NdeI* and *BamHI* and ligating the *lipC12* coding region into the pET29a vector (Novagen, Hertfordshire, United Kingdom), which
had been previously digested with the same restriction enzymes. The resulting plasmid was called pET29lipC12. It was used to produce recombinant LipC12 without a His-tag (denominated rLipC12).

Additionally, the lipC12 gene was cloned into the pTEV5 vector to obtain the plasmid pTEV5lipC12. This was done by digesting pET28lipC12 with NdeI and BamHI and ligating the lipC12 coding region into the pTEV5 vector, which had been previously digested with the same restriction enzymes. The LipC12 produced in cells harboring pTEV5lipC12 is named HisTEV-LipC12 and contains a His-tag at the N-terminal and a TEV protease site (TEVS) to allow His-tag cleavage with TEV. After TEV cleavage, the protein is named cLipC12, which is an un-tagged LipC12 that contains 4 extra amino acids (GASH).

His-LipC12, rLipC12, HisTEV-LipC12 and TEV protease were overexpressed in Escherichia coli strains harboring the plasmids listed in Supplementary Table S2. The E. coli strains and expression conditions are also described in Supplementary Table S2. The overexpression and cell disruption by ultrasonication were done as previously described15,16.

Protein purification. HisTEV-LipC12 was purified using a 5-mL HiTrap Chelating column (GE Healthcare, Uppsala, Sweden) preequilibrated with buffer A (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 10% (v/v) glycerol and 20 mM imidazole). The protein was eluted stepwise, using increasing concentrations (from 50 to 500 mM) of imidazole in buffer A, with 2 column volumes of buffer being passed at each concentration. Fractions with purity over 95%, as judged by SDS-PAGE (Supplementary Fig. S1), were pooled. Imidazole was removed in a HiTrap Desalting column (GE Healthcare, Uppsala, Sweden) using a 20-mM Tris-HCl buffer at pH 7.5 that contained 150 mM NaCl and 5% (v/v) glycerol. TEV protease was purified as previously described12.

Cleavage of the His-tag with TEV protease. Purified HisTEV-LipC12 and TEV protease were mixed in 2 mL of buffer (20 mM Tris-HCl pH 7.5 and 150 mM NaCl), in a mass ratio of HisTEV-LipC12 to TEV protease of 10:1. The mixture was maintained at 8°C, 90 rpm for 20 h. It was then passed through a 5-mL HiTrap Chelating column (GE Healthcare). cLipC12 was eluted with 100 mM imidazole and TEV protease was eluted with 300 mM imidazole. Imidazole was removed from the LipC12 preparation in a desalting column, using a 20 mM Tris-HCl buffer at pH 7.5 that contained 150 mM NaCl and 5% (v/v) glycerol. TEV protease was purified as previously described12.

Titrimetric determination of triacylglycerol-hydrolyzing activity. The triacylglycerol-hydrolyzing activities of the soluble fractions of crude extracts and of purified lipase preparations were determined by the titrimetric method using an automatic titrator (Metrohm 718 STAT Titrisco potentiometric titrator, Metrohm, Herisau, Switzerland) with 0.05 M NaOH during 5 min. The conditions have been previously described16,43. The reactions were performed at 30 °C using 20 mL of emulsion at pH 7.0. One unit of hydrolytic activity in aqueous medium corresponds to the release of 1 μmol of fatty acid per min, under the assay conditions.

Spectrophotometric determination of pNPD-hydrolyzing activity. The pNPD-hydrolyzing activities of the lipases in aqueous solution were determined by monitoring the release of p-nitrophenol from p-nitrophenyl decanoate (Sigma, St. Louis, USA) at 410 nm at 25 °C in an iMark ™ Microplate Absorbance Reader (Bio-Rad, Hercules, USA).

The standard assay conditions have been previously described15. The final volume of the reaction mixture was 250 μL. It contained 50 mM Tris-HCl buffer, pH 7.5, 1 mM CaCl₂, 0.6% (v/v) Triton X-100, 1 mM pNPD, 4% (v/v) isopropanol, 1% (v/v) acetonitrile and an enzyme concentration of 172 nM when HisTEV-LipC12 was assayed and of 123 nM when LipC12 was assayed. One unit of lipase activity was defined as 1 μmol of p-nitrophenol produced per min. Linear regressions to determine initial reaction velocities were performed with Microsoft Excel.

The pNPD-hydrolyzing activities of the lipases in the presence of polar organic solvents were determined at concentrations varying from 10 to 60% (v/v) of the solvent (ethanol, isopropanol, methanol, propanol, acetone, acetonitrile, dimethylformamide (DMF) or dimethylsulfoxide (DMSO)) in water. The activity was determined in a 96-well plate by monitoring the release of p-nitrophenol from pNPD, according to standard assay conditions with the addition of the stated solvent concentrations.

The stability of the lipases was determined by measuring the residual activity after incubation (at final concentrations of 1.72 μM for HisTEV-LipC12 and 1.23 μM for LipC12) for 3 h at 25 °C in the same polar organic solvents described for activity. The enzymes were then diluted in 50-mM Tris-HCl buffer at pH 7.5, 150 mM NaCl and 2-mM CaCl₂ in the reaction media to reach the protein concentrations of 172 nM and 123 nM used in the standard assay of pNPD-hydrolyzing activities.

Determination of kinetic parameters. The kinetic parameters were determined using concentrations of pNPD ranging from 0.1 to 3.5 mM and 10 μL of enzyme (enzyme concentration in the reaction medium of 6.9 μM for HisTEV-LipC12 and 4.9 μM for LipC12). The reaction was followed for 5 min with the absorbance at 410 nm being read every 30 s. The values of $K_{m}$ and $V_{max}$ were determined by non-linear regression, in GraphPad Prism 7, of the data of initial velocity versus substrate concentration, using the Michaelis-Menten equation. The program was used to calculate 95% confidence intervals.

Circular dichroism. CD spectra of proteins were recorded between 250 and 350 nm using a JASCO J-815 spectropolarimeter (Easton, USA) fitted with a Jasco Peltier-type temperature controller (CDF-4265/15). The optical pathlength was 2 mm and the concentration of the protein solution was 5 μM in a pH 7.4 buffer containing 10 mM sodium phosphate and 100 mM NaCl. The spectra were recorded at 25 °C. The percentages of secondary structures (α-helices and β-sheets) were determined with the software K2D344, after conversion of the data to delta epsilon values (Δε), also referred as the molar circular dichroism45.
The melting temperatures (Tm) were determined by measuring the CD signal at 222 nm with increasing temperature in a continuous ramp mode (1 °C/2 min). The temperature was increased from 25 to 98 °C. The Tm was determined from the first derivative using GraphPrism 6.0.

**Lipase immobilization.** The covalent immobilization of LipC12 preparations was performed using Immobeads 150 (Sigma–Aldrich, St. Louis, USA) according to Madalozzo et al. The immobilization kinetics of crude soluble fractions containing His-LipC12 and cLipC12 were determined with protein loadings of 200 mg g⁻¹ during 8-h incubations at 4 °C in an orbital shaker at 150 rpm. Purified Histagged-LipC12 and cLipC12 were also immobilized on Immobead 150 with protein loadings of 10 mg g⁻¹, with incubation for 6 h at 4 °C in an orbital shaker at 150 rpm. A desorption test was done after immobilization. Twenty mg of the support was added to 200 μL of a 2% (m/v) SDS solution. The mixture was heated at 100 °C for 30 min. After that, samples were loaded onto a 12% SDS-PAGE gel for analysis. The percentage of desorption was calculated based on the protein contents of the samples (which were estimated using the protein standards supplied by the manufacturer, GE Healthcare).

**Determination of the esterification activity.** The esterification activities of the immobilized preparations of LipC12 were determined based on ethyl-oleate synthesis. The reactions were carried out in 25-mL Erlenmeyer flasks, with 5 mL of reaction medium (210 mmol L⁻¹ ethanol and 70 mmol L⁻¹ oleic acid in n-hexane). The flasks were placed in an orbital shaker at 40 °C and 250 rpm. The reaction was started with the addition of 110 mg of the immobilized enzyme. At each sampling time, a 100-μL sample was collected and analyzed to quantify free fatty acids by the colorimetric Lowry-Tinsley method. One unit of lipase esterification activity (U) corresponds to the consumption of 1 μmol of fatty acid per min, under the assay conditions.

**Protein analysis.** The molecular weights of the proteins were determined using the ProtParam tool of the online program Expasy. Protein electrophoresis was carried out by 12% SDS-PAGE and gels were stained with Coomassie Blue. Densitometry analysis of the protein bands from the SDS-PAGE gel was performed using the online program Expasy. Protein electrophoresis was carried out by 12% SDS-PAGE and gels were stained with Coomassie Blue. Densitometry analysis of the protein bands from the SDS-PAGE gel was performed using the online program Expasy. Protein electrophoresis was carried out by 12% SDS-PAGE and gels were stained with Coomassie Blue. Densitometry analysis of the protein bands from the SDS-PAGE gel was performed using the online program Expasy. Protein electrophoresis was carried out by 12% SDS-PAGE and gels were stained with Coomassie Blue. Densitometry analysis of the protein bands from the SDS-PAGE gel was performed using the online program Expasy. 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Author Contributions
J.M.A and V.R.M. designed, performed the experiments, analyzed the data, wrote and revised the manuscript. D.A.M. and N.K. contributed to data analysis, wrote and revised the manuscript. M.M.S., E.M.S. and F.O.P reviewed the manuscript.

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Competing Interests
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