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Semisynthetic peptide–lipase conjugates for improved biotransformations†

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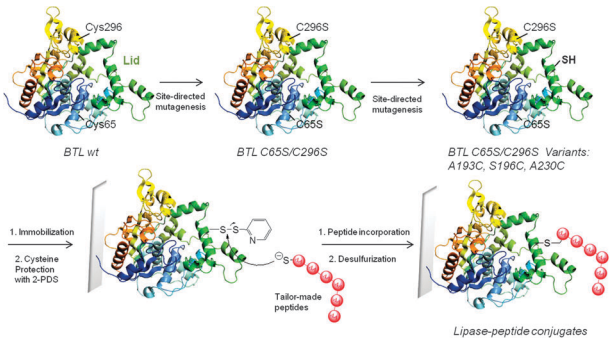
An efficient chemoselective method for the creation of semisynthetic lipases by site-specific incorporation of tailor-made peptides on the lipase-lid site was developed. These new enzymes showed excellent improved specificity and regio- or enantioselectivity in different biotransformations.

Chemical modification is a fascinating approach for altering the protein function by the introduction of non-natural fragments into proteins.[1] The addition of unnatural moieties (such as fluorophores, sugars, peptides, *etc.*) to proteins[2] has also proven useful for a variety of processes and applications both *in vivo* and *in vitro*. Furthermore this specific modification on proteins has permitted the alteration of catalytic properties of enzymes for creation of novel active selective biocatalysts.[3,4] Therefore the combination of molecular biology methods and efficient synthetic approaches has made possible successful preparation of semisynthetic proteins in large amounts.[5,6] Modification of the nucleophilic thiol of a unique cysteine is a widely employed strategy for site-selective bioconjugation.[7] A cysteine can be introduced at virtually any position within a protein structure by site-directed mutagenesis and then selectively modified using for example disulfide compounds. Furthermore, the disulfide can be efficiently transformed into a thioether by a desulfurization method.[8]

Herein, we describe the design of new semisynthetic enzymes by the site-specific incorporation of a set of tailor-made cysteine-containing peptides on successfully engineered thermophilic lipase from *Geobacillus thermocatenulatus* (BTL) with a single cysteine at different positions on the lid-site of the enzyme (Scheme 1). The chemical modifications were performed on the lipase already immobilized on CNBr-activated Sepharose.



Scheme 1 Lipase-conjugates preparation.

This introduces the advantages of solid-state chemistry: using an excess of peptides, quantitative transformations, or easy purification.[9] The catalytic activity of the new semisynthetic enzymes in different asymmetric biotransformations was evaluated.

The incorporation of molecules on the oligopeptide lid represents an elegant strategy to control its movement and therefore the lipase catalysis. As far as we know, no example of the improvement of lipase catalytic efficiency by this methodology has been described.

First we focused on the protein engineering of this lipase to replace the two cysteines (Cys65, Cys296) in the wild type enzyme (BTL wt) by two serines. The new-engineered enzyme (BTL C65S/C296S) was expressed in *E. coli* without detriment to the enzyme activity. Considering the complexity of this lipase with a tricky lid formed by two different loops, the idea was to introduce a unique cysteine at different positions of this engineered enzyme. This approach was first confirmed by a bioinformatics study using the crystallographic open conformation of the enzyme we have recently solved.[10]

Three different positions were selected to be mutated: Ala193 (in the internal loop), Leu230 (in the external loop) and Ser196 (in the middle of both loops). These three variations represent a conservative change. The three new mutants BTL-C65S/C296S/A193C (BTL*-A193C), BTL-C65S/C296S/L230C (BTL*-L230C) and BTL-C65S/C296S/S196C (BTL*-S196C) were obtained by site-directed mutagenesis with a good production, similar to BTL wt or BTL C65S/C296S. All BTL variants were efficiently purified by hydrophobic chromatography[10] and characterized by circular dichroism and fluorescence (see ESI†, Fig. S1 and S2).

For the strategic chemical incorporation of tailor-made peptides including a cysteine on the peptide sequence, the

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protein cysteine was previously protected using 2,2′-dithiodipyridine disulfide (2-PDS) to avoid oxidation (Scheme 1).[4] The different protected BTL variants were specifically modified by disulfide exchange. Considering the amino acid residues on the lid loops two different peptides Ac-Cys-Phe-Gly-Phe-Gly-Phe-$CONH_2$ (**p1**) and Ac-Cys-$(Asp)_4$-Asp-COOH (**p2**) were selected (Fig. S3, ESI†). The BTL*-A193C was modified with the complementary **p1**, BTL*-L230C with **p2** whereas BTL*-S196C was modified with **p1** or **p2**. The coupling reaction in all cases was performed in aqueous solution at pH 8 and 25 °C (see ESI†). After coupling the desulfurization was performed[8] to get a thioether irreversible bond between the enzyme and the peptide. A quantitative conversion into the corresponding peptide-modified enzymes was observed by mass spectroscopy (MALDI-TOF; Fig. 1B; Fig. S4 and S5, ESI†). The excess of peptide was easily removed by simple filtration of the immobilized semisynthetic lipases. No enzyme modification was observed after treatment of the BTL-C65S/C296S variant with the peptides. The circular dichroism (CD) spectra confirmed that the incorporation of the peptide affected the secondary structure of the enzyme with increase in alpha-helix structure after modification (Fig. 1C; Fig. S4 and S5, ESI†). The fluorescence results (Fig. 1D; Fig. S4 and S5, ESI†) show a decrease in signal intensity by the peptide coupling. The irreversible inhibition experiments (Fig. S6, ESI†) showed that the BTL variants exhibited a more open conformation with the peptide incorporation.

In order to elucidate structural changes caused by peptide incorporation, molecular dynamics simulations were carried out for BTL*-A193C–**p1** and BTL*-S196C–**p2** variants. As observed in crystal structures, BTL activation involves large structural rearrangements and the concerted movement of two lids, L_1 and L_2 (Fig. 2), unmasking the active site.[10] Conformational transitions from the closed to the open state of BTL were simulated by the Targeted Molecular Dynamics (TMD) method, based on the crystal structures of both states (see ESI†, Fig. S7). TMD coupled with energy minimizations clearly indicate the resulting active sites that could be reached in BTL variants depending on both the nature of the peptide stem and the position at which they are attached. In both

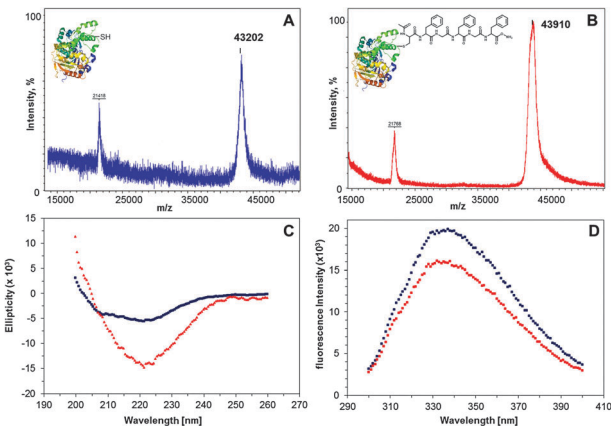


Fig. 1 Characterization of the BTL*-A193C–**p1** conjugate. (A) MALDI-MS spectra of BTL*-A193C, [M + Na]; (B) MALDI-MS spectra of BTL*-A193C–**p1**, [M + Na]; (C) far-UV CD spectra; (D) fluorescent spectra. BTL*-A193C (blue), BTL*-A193C–**p1** (red).

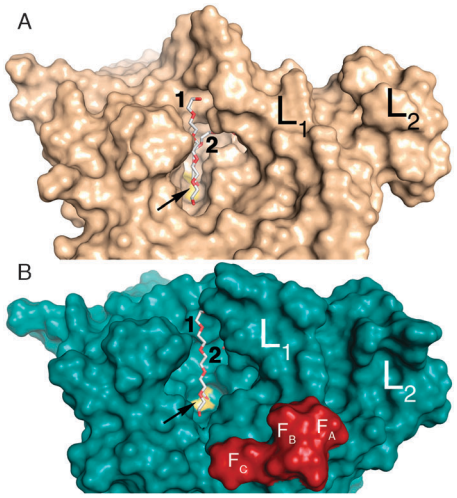


Fig. 2 Comparison between crystal structures of active BTL and a model active BTL*-A193C–**p1** variant. (A) Molecular surface of active BTL with Triton X-100 moieties in the active site (sticks 1 and 2). Lids L1 and L2 are labeled. (B) Molecular surface of BTL*–A193C–**p1**. Detergent moieties from the crystal structure are superimposed for comparison. As observed, there is no room for moiety 2. Arrows indicate the position of the catalytic Ser114. The position of **p1** is highlighted in red, and the aromatic residues (F_A, F_B, and F_C) are labeled.

variants, computer simulations indicate a smaller active site of the modified enzyme than the wild type enzyme. The model of the active BTL*-A193C–**p1** variant reveals that the **p1** moiety lies on a hydrophobic patch at the active site (Fig. 2 and Fig. S8, ESI†). The active site of the BTL*-S196C–**p2** variant presents strong modifications compared with that of BTL. This is mainly due to the insertion of the acidic **p2** moiety between L_1 and L_2 lids (Fig. S9, ESI†) that establishes strong salt bridge interactions with basic residues of both lids (Fig. S10, ESI†).

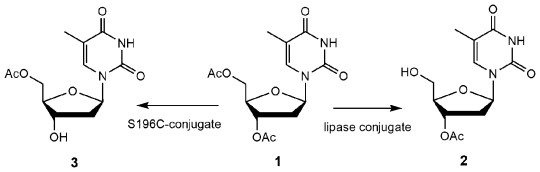
The method was extended to peptides **p3–p8** [Ac-Phe-Cys-Phe-Gly-Phe-$CONH_2$ (**p3**), Ac-Gly-Gly-Cys-Gly-Gly-$CONH_2$ (**p4**), Ac-Cys-$(Arg)_7$-$CONH_2$ (**p5**), Ac-Cys-(Phe-Gly-)$_2$-Asp-Asp-$CONH_2$ (**p6**), Ac-Asp-Gly-Asp-Cys-Asp-$CONH_2$ (**p7**), Ac-Lys-Gly-Lys-Cys-Lys-$CONH_2$ (**p8**)] (Fig. S11 and Table S2, ESI†) with amino acids with different properties affording the desire conjugates with high selectivity in yields >95%.

In order to investigate the catalytic effect of the peptide incorporation on the BTL variants, these new biocatalysts were applied in three different biotransformations. BTL wt and BTL C65S/C296S variants showed similar catalytic results in all biotransformations tested (see ESI†).

In the regioselective deprotection of per-*O*-acetylated thymidine (**1**) (Table 1), the BTL*-A193C and BTL*-L230C variants (mutants on both loops) hydrolyzed mainly at the *C*-5 position of ribose with slightly higher regioselectivity than BTL C65S/C296S. The incorporation of **p1** in the BTL*-A193C or **p2** in the BTL*-L230C variant improved the regioselectivity of the enzyme (around 88% of **2**) (Table 1). However, the incorporation of other peptide sequences did not produce a better result on regioselectivity (Tables S3 and S4, ESI†).

The introduction of the cysteine at 196 (BTL*-S196C) caused an inversion and good regioselectivity of the lipase,

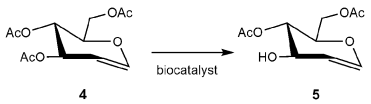
Table 1 Regioselective hydrolysis of peracetylated tymidine **1** catalyzed by peptide-modified immobilized BTL2 variants



Enzyme	Peptide	pH	Time [h]	Yield[a] **2** [%]	Yield[a] **3** [%]	T
BTL C65S/C296S	—	7.0	70	52	5	43
BTL*-A193C	—	7.0	93	79	8	13
BTL*-A193C	**p1**	7.0	48	88	4	8
BTL*-S196C	—	5.0	50	0	86	14
BTL*-S196C	**p2**	5.0	100	0	97	3
BTL*-L230C	—	7.0	57	61	14	25
BTL*-L230C	**p2**	7.0	93	86	4	10

[a] Yield of the corresponding product at 100% conversion. T: thymidine.

Table 2 Regioselective hydrolysis of peracetylated glucal **4** catalyzed by peptide-modified immobilized BTL2 variants



Enzyme	Peptide	Time [h]	Activity[a]	Yield[b] **5**[%]
BTLC65S/C296S	—	24	5	79
BTL*-A193C	—	1	183	70
BTL*-A193C	**p1**	1	214	73
BTL*-A193C	**p3**	5	40	94

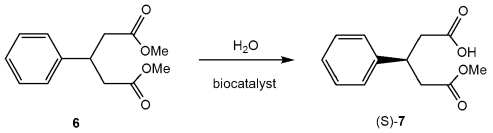
[a] Specific activity is defined as: μmol min^{-1} mg_{lip}^{-1} $\times 10^{-3}$. [b] Yield of the monodeprotected **5** at 100% conversion. The rest of yield corresponds to the bihydrolyzed product.

hydrolyzing at the *C*-3 position of **1** (86% of **3**) without any trace of **2** (Table 1). The incorporation of **p2** enhanced the regioselectivity of this BTL variant up to an excellent yield (97% of **3**) with no trace of **2**. This is the best value in enzymatic regioselectivity ever reported in the literature so far. The BTL*-A193C and BTL*-L230C peptide-conjugates showed lower specificity when the reaction was performed at pH 5.0 (Table S3, ESI†) whereas the BTL*-S196C variant showed very low specificity at pH 7.0 (Table S4, ESI†).

Secondly, the regioselective deprotection of per-*O*-acetylated glucal (**4**) was successfully catalysed by the semisynthetic enzymes (Table 2 and Table S5, ESI†). All BTL variants were regioselective to the monodeprotection at *C*-3. Surprisingly, an increase of more than 36 fold of the enzyme activity was observed with the introduction of the cysteine at 193. The incorporation of **p1** increased the activity but not the regioselectivity whereas **p3** in BTL*-A193C improved the yield of **5** from 70 to 94%. For the other two mutants, the regioselectivity was not improved after the enzyme–peptide conjugates formation (Table S5, ESI†).

Finally, the catalysis of the desymmetrization of dimethylphenylglutaric acid diester (**6**) was studied. The three-monocysteine

Table 3 Desymmetrization of phenylglutaric acid dimethyl ester catalyzed by different BTL-variants at pH 7.0



Enzyme	Peptide	Time [h]	*C* [%]	Yield[a] [%]	ee[b] [%]
BTL-C65S/C296S		196	28	23	64
BTL*-A193C	—	186	23	15	78
BTL*-A193C	**p1**	96	54	47	>99

[a] Yield of the monoester **7**. The rest of conversion corresponds to the dicarboxylic acid. [b] Determined by HPLC.

BTL variants showed higher enantiomeric excess than BTL wt and BTL C65S/C296S (*e.g.* from 64% to 78%) (Table 3 and Table S8, ESI†). The best result was found after the chemical modification of BTL*-A193C with **p1**. This semisynthetic lipase showed higher activity (4 fold) and excellent enantiomeric excess (>99%). The peptide modification also increased the selectivity of BTL*-S196C (with **p1** or **p2** ee >99%) although a negative effect was observed for BTL*-L230C (Table S6, ESI†).

In conclusion, we report for the first time an efficient chemoselective, and potentially general method, for the creation of semisynthetic lipases by site-specific chemical incorporation of tailor-made peptides on the lid-site of three different cysteine-BTL variants based on a fast thiol-disulfide exchange ligation.

These modifications generated new enzyme variants showing different structural changes in the lid region yielding important changes in the catalytic properties.

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