Protein Engineering

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Creation of a Lipase Highly Selective for *trans* Fatty Acids by Protein Engineering

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During the past two decades, the consumption of *trans* fatty acids (TFA) has been identified as an important modifiable risk factor for coronary heart disease and has been linked to the aggravation of visceral adiposity and insulin resistance in predisposed individuals.[1] Multifaceted political and legislative actions have therefore been undertaken to restrict the use of TFA in food formulations in most Western countries. Most of these TFAs form as undesired by-products in the industrial hydrogenation of polyunsaturated vegetable oils, a process that aims to improve the oxidative stability and melting-point properties of plant oils. Since its invention by Wilhelm Norman in 1902,[2] catalytic hydrogenation has remained the predominant process for the modification of edible fats. Around 5% of the global production of vegetable oils (totaling approximately 137 million metric tons per year[3a]) is still subjected to hydrogenation, although alternatives such as fractionation and interesterification have gained more relevance in the production of "zero *trans*" edible fats.[3] Consequently, the selective removal of *trans* fatty acids from partially hydrogenated vegetable oils (PHVOs) would be a highly valuable downstream process for industrial hydrogenation. Unfortunately, neither chemical nor enzyme catalysts that enable the highly selective removal of TFAs from PHVO have been described. It was reported, however, that lipase A from *Candida antarctica* (CAL-A) exhibits a 15 times higher reaction rate for the esterification of elaidic acid (C18:1Δ9-*trans* fatty acid) with *n*-butanol over the analogous esterification of oleic acid (C18:1Δ9-*cis* fatty acid).[4] Until now, the reasons for this *cis/trans* selectivity have not been elucidated and hence a model for this type of fatty acid selectivity is lacking.

Like all lipases, CAL-A possesses the typical α/β-hydrolase fold, and the active site contains a triad consisting of serine, histidine, and aspartate that enables the hydrolysis of water-insoluble esters or the reverse reaction (esterification).[5] CAL-A is a peculiar lipase with interesting features; it accepts very bulky substrates including highly branched acyl groups and sterically hindered alcohols, amines, and amides.[6] After the crystal structure of CAL-A was solved by Ericsson et al. in 2008,[7] protein engineering of CAL-A was successfully performed to improve its enantioselectivity towards sterically demanding substrates.[8] Furthermore, the X-ray structure of CAL-A (PDB entries: 2VEO and 3GUU) revealed an atypical lid structure, suggesting that CAL-A is a prototype of a new lipase subfamily. This unique lid creates a very long and narrow acyl-binding tunnel, plus a small appendix, believed to provide long-chain fatty acids an excellent fit within the CAL-A structure.

We thus used CAL-A as a starting point, aiming to create a highly *trans*-selective variant for the efficient removal of TFA from PHVO. This required: 1) functional expression of CAL-A in *Escherichia coli* to facilitate a microtiter plate (MTP) based assay for high-throughput screening (HTS), 2) a suitable HTS method for screening mutant libraries, and 3) the identification of putative amino acid residues with an influence on *trans* or *cis* fatty acid selectivity as targets for mutagenesis.

To improve protein production on MTP and deep-well-plate (DWP) scales, four genetic constructs were created, as the previously reported expression of CAL-A[9] in *Pichia pastoris* and *E. coli* was found to be not optimal for this task. We found that the periplasmic expression of CAL-A in *E. coli* led to significantly higher activities in the culture supernatant than cytoplasmic expression. Propitiously, self-lysis of the cells caused the lipase to accumulate in the supernatant and hence a time-saving and convenient method was established (see Figures S1 and S2 in the Supporting Information). Next, we developed a system for a high-throughput assay. This was based on the release of *p*-nitrophenol from esters of either elaidic acid (*p*NP-C18:1Δ9 *trans*) or oleic acid (*p*NP-C18:1Δ9 *cis*) (Scheme 1) as these are the most abundant unsaturated fatty acids in PHVO. With this pair of representative *trans* and *cis* fatty acids we could determine the *trans*-over-*cis* selectivity (ToC value) of wild-type CAL-A or mutants by following the rate of hydrolysis of the *p*NP esters in separate wells of a MTP. Enzyme variants with the desired improved selectivity were then subjected to detailed analysis using PHVO and GC analysis.

Using this assay, a ToC value of wild-type CAL-A of 2.5 was determined for elaidic/oleic acid. The difference in selectivity to that reported earlier[4] can be attributed to the reaction system (esterification vs. hydrolysis) and the differing ester (*p*NP vs. *n*-butyl esters). In addition, the vaccenic acid ester (*p*NP-C18:1Δ11 *trans*) was synthesized and a ToC value of 3.0 was determined (see Figure S3 in the Supporting Information).

The identification of residues involved in the selectivity of CAL-A towards the *trans* or *cis* configuration of a double bond in a fatty acid is challenging and has never been addressed in protein engineering so far. Hence, all residues comprising the roughly 30 Å long acyl-binding tunnel of

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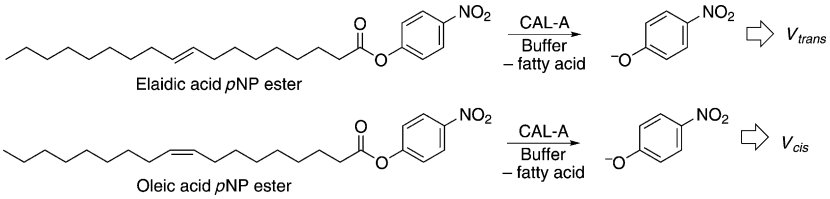

Scheme 1. The hydrolysis of *p*-nitrophenyl esters (*p*NPs) of elaidic acid (*trans*) or oleic acid (*cis*) by CAL-A or its mutants releases *p*-nitrophenol, which was spectrophotometrically quantified. The rate of hydrolysis was used to calculate the ToC value ($ToC = v_{trans}/v_{cis}$).

CAL-A were considered, specifically residues within a 5 Å radius and facing the docked C18:1 fatty acid (Figure 1). This led to the identification of twelve positions along the entire fatty acid binding tunnel: F149, I150, A218, T221, F222, L225, F233, G237, L241, M248, I301, and L305. Thus, not only residues near the likely position of the Δ9 double bond, but also amino acids at the beginning and the end of the acyl-binding tunnel were subjected to mutagenesis. The NDT codon was chosen for site-directed saturation mutagenesis as it encodes for only 12 of the 20 proteinogenic amino acids, but covers all types of amino acid properties. Consequently, fewer clones needed to be screened for each position without compromising the quality of the library.[10]

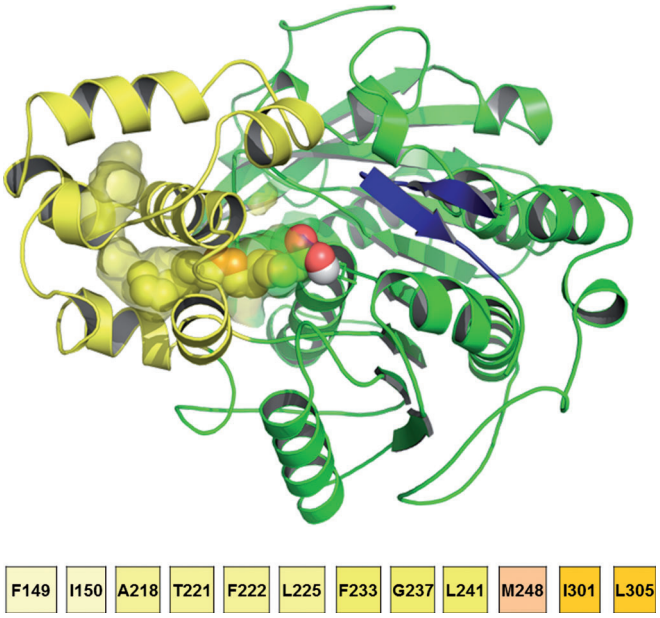

Figure 1. Crystal structure of CAL-A (PDB entries: 2VEO, 3GUU). A C18:1 fatty acid is docked into the acyl-binding tunnel of CAL-A. The twelve residues chosen for NDT-codon-based saturation mutagenesis are indicated below.

After creation of the twelve NDT-codon-based libraries and expression of the variants in *E. coli* in DWP, the libraries (ca. 5000 clones) were screened using the assay shown in Scheme 1. Mutagenesis of seven out of the twelve positions (I150, A218, L225, F233, G237, L241, M248) led to variants with ToC values lower than or similar to that of wild-type CAL-A. For positions F149, T221, F222, I301, and L305, however, several variants with two- to sixfold higher ToC values were identified (Table 1) even if the specific activity was sometimes reduced (i.e. for T221H, F222C, and I301H). Of particular note, mutants F149D and F222S demonstrated only a moderately impaired hydrolysis rate and a strongly increased preference for the *trans* isomer.

Next, the relevance of these increased ToC values was verified in an assay representative of the desired application of the enzyme, specifically, the hydrolysis of PHVO, a complex mixture of triglycerides containing over 70 saturated and unsaturated fatty acids with differing chain lengths and numbers and positions of double bonds. The specific PHVO used in this study (partially hydrogenated soybean oil; PHSO) contains around 20 different *trans* fatty acids resembling about 18% of the total fatty acids in the oil. Thus, ToC values determined with the *p*NP esters may differ in the hydrolysis of this complex oil. Large-scale production of the six best CAL-A variants was performed using *P. pastoris* X33 or *E. coli* (DE3). The culture supernatant was then used in the hydrolysis of PHSO followed by GC analysis of the fatty acids that remained bound to glycerol. Instead of the ToC value described above, an alternative criterion was needed to judge the chemoselectivity of the mutants in the hydrolysis of the highly complex PHSO as various *trans*, *cis*, and saturated fatty acids are present in the oil. Therefore a selectivity factor (α_G factor) was introduced reflecting the chemoselectivity of the CAL-A mutants for different types of fatty acids (e.g. *trans* and saturated vs. *cis*). This expanded α factor is identical to the E value introduced to judge the conversion-independent enantioselectivity as defined by Chen et al.[11] or to the competition factor developed by Rangheard et al. for multicompetitive enzymatic reactions.[12]

Thus, a high α_G factor reflects an enzyme with a high preference for the hydrolysis of *trans* or saturated fatty acids. Table 2 shows that indeed all variants were more selective than wild-type CAL-A, with T221H and I301H being the most selective mutants.[13] A detailed analysis of the fatty acids released by each variant compared to those released by wild-type CAL-A can be found in the Supporting Information (Figure S4, Table S1). We found that each CAL-A variant

Table 1: Best mutants found in the initial screening of CAL-A variants with *p*NP esters of elaidic acid and oleic acid.

CAL-A variant	ToC value	v [μmol min^{-1}] C18:1Δ9 *trans*[a]			v [μmol min^{-1}] C18:1Δ9 *cis*[b]		
WT	2.5	14188	±2838	(100%)	5675	±1135	(100%)
I305N	4	2696	±539	(19%)	454	±91	(8%)
T221H	5	709	±142	(5%)	114	±23	(2%)
I301H	6	1135	±227	(8%)	170	±34	(3%)
F222C	8	1135	±227	(8%)	114	±23	(2%)
F149D	12	2412	±482	(17%)	170	±34	(3%)
F222S	15	2979	±596	(21%)	170	±34	(3%)

[a] Enzymatic rates of *p*NP ester hydrolysis of CAL-A mutants and relative activity of mutants compared to activity of WT.

Table 2: Hydrolysis of PHVO using different CAL-A variants.

CAL-A variant	Conv. [%]	Act. Σ*cis* [mU mL^{-1}][a]	Act. Σ*trans* [mU mL^{-1}][a]	Act. Σsat [mU mL^{-1}][a]	$\nu\Sigma trans/\nu\Sigma cis$[b]	$\nu\Sigma sat/\nu\Sigma cis$[b]	α_{G1}[c]	α_{G2}[d]
wild-type	22	883	1203	1076	1.36	1.22	3.5	6.7
L305N	14	120	299	239	2.50	2.00	4.5	9.6
F149D	11	3	78	74	27.77	26.20	4.6	28.5
F222S	16	16	111	117	7.02	7.37	4.5	32.2
F222C	20	46	163	164	3.53	3.54	4.6	36.6
T221H	5	0	48	47	>100	>100	9.8	>100
I301H	12	0	107	87	>100	>100	11.2	>100

[a] Volumetric activity of CAL-A variants for released fatty acids (Σ*cis:* released amount of *cis* fatty acids, Σ*trans*: released amount of *trans* fatty acids, Σsat: released amount of saturated fatty acids). One unit is defined as the amount of enzyme that releases one μmol of fatty acids per minute under the assay conditions. [b] Rates for release of *trans* fatty acids (Σ*trans)* or saturated fatty acids (Σ sat) vs. rate of release of *cis* fatty acids (Σ*cis*). [c] α_G factor for specificities $\Sigma trans_{bound}/(\Sigma cis_{bound}+\Sigma sat_{bound})$; [d] α_G factor for specificities: $(\Sigma trans_{bound}+\Sigma sat_{bound})/\Sigma cis_{bound}$; $\Sigma trans_{bound}$: bound fraction of *trans* fatty acids; Σcis_{bound}: bound fraction of *cis* fatty acids; Σsat_{bound}: bound fraction of saturated fatty acids in PHVO. All measurements were made in triplicate.

exhibited an individual profile of fatty acid hydrolysis rates (Table S1) suggesting that each substitution in the acyl-binding tunnel of CAL-A can lead to a uniquely tailored set of fatty acid preferences. Furthermore, both best CAL-A variants gave an excellent α_{G2} factor exceeding 100, meaning that *trans* and saturated fatty acids are preferentially hydrolyzed from PHVO. This provides an additional important health benefit to the resulting oil, as only the *cis* fatty acids remain bound in the glyceride fraction.

In addition, the analysis of the hydrolysis data (Table 2) indicates that the improved CAL-A variants do not differentiate between saturated and *trans* fatty acids, but primarily between those that can adopt linear geometries (saturated and *trans* fatty acids) and those that cannot (*cis* fatty acids). The straight and narrow tunnel of the *trans*-selective CAL-A (Figure 1) may hinder the access of *cis* fatty acids while allowing *trans* and saturated fatty acids to enter. By this mechanism, a narrow region in the binding tunnel will impart a general specificity against *cis* double bonds at any position on the fatty acid deeper in the tunnel than the narrow region. Conversely, *cis*-selective enzymes (such as *G. candidum* lipase I[14]) may operate through tight arches restricting access to long, straight *trans* fatty acids.

In conclusion, our novel approach to engineer the *trans* fatty acid selectivity of a lipase enabled the efficient alteration of the chemoselectivity of CAL-A leading to variants with the desired and excellent properties. In the future, therefore, application of the best variants to the large-scale processing of partially hydrogenated plant oil may help prevent exposure of humans to unhealthy *trans* fatty acids.

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