Enzymes in lipid modification: From classical biocatalysis with commercial enzymes to advanced protein engineering tools

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Abstract: *In this review, the application of enzymes, especially lipases, for the modification of fats and oils is covered. This includes the lipase-catalyzed selective production of structured triglycerides and the isolation or incorporation of specific fatty acids. Protein engineering methods to modify lipases on a molecular level were used to alter the fatty acid chain-length and ''trans over cis'' selectivity of lipase A from Candida antarctica. Furthermore, an enzymatic cascade reaction to remove 3-monochloropropanediol and the identification of a phospholipase C for degumming are briefly covered.*

Key words: *lipase, phospholipase, biocatalysis, lipid modification, structured triglycerides, degumming, 3-MCPD, protein engineering*

Introduction

A wide range of enzymes can be used for the modification of fats, oils and other lipids. This is due to their usually excellent chemo-, regio- and stereoselectivity and especially the mild reaction conditions compared to chemical methods. Commonly used enzymes are first of all lipases, but also various phospholipases or lipoxygenases have been investigated and are applied in lipid modification. The application of biocatalysts is well documented in the literature (Biermann *et al.*, 2011; Bornscheuer, 2000; Bornscheuer, 2003; Bornscheuer *et al.*, 2012; Metzger and Bornscheuer, 2006; Schörken and Kempers, 2009) and will therefore not be covered in detail here. This article rather focuses on achievements of my own research as presented at the Journées Chevreul conference in Paris.

Lipase-catalyzed synthesis of structured triglycerides

The by far most often used biocatalysts in lipid modification are lipases (EC 3.1.1.3, triacylglycerol hydrolases) as fats and oils are their natural substrates (Schmid and Verger, 1998; Jaeger, 1994). These enzymes do not require cofactors, many of them are available from commercial suppliers and they exhibit high activity and stability, even in non-aqueous environments. Because lipases show distinct chemo-, and regioselectivity, they can be used for the tailoring of natural lipids to meet nutritional properties, especially for humans. One example is the industrial synthesis of cocoa-butter equivalent, which is predominantly 1,3-disaturated-2-oleyl-glyceride. Processes had been developed by Unilever and Fuji Oil with 1,3-regioselective lipases for acidolysis of cheap plant oils using stearic acid as acyl donor.

Structured triglycerides (sTAG) such as cocoa-butter equivalent have a defined distribution of different fatty acids along the glycerol backbone and are important compounds for a range of applications in human nutrition. sTAG containing medium chain fatty acids at the *sn*1- and *sn*3-position together with a long (preferentially polyunsaturated fatty acid) at the *sn*2-position such as 1,3-caproyl-2-oleyl-glycerol (CyOCy) are used, for instance, to treat patients with pancreatic insufficiency as well as for rapid energy supply (i.e., for sports). Another important example is Betapol™ used in infant nutrition (Kennedy, 1999). It contains oleic acid at the *sn*1- and *sn*3-position and palmitic acid at the *sn*2-position (1,3-oleoyl-2-palmitoyl-glycerol, OPO). Betapol™ is manufactured by interesterification of tripalmitin with high oleic sunflower oil

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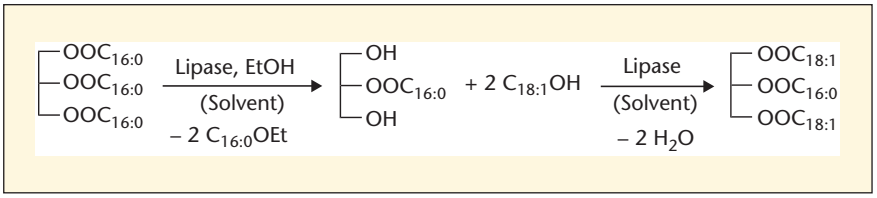

Figure 1. *Structured triglycerides were obtained in high yield and purity in a two-step enzymatic process. First tripalmitin is subjected to lipase-catalyzed alcoholysis with ethanol yielding sn2-monopalmitin, which can be isolated by crystallization. In the second step this monoglyceride is esterified with oleic acid yielding the desired sTAG such as Betapol.*

using a lipase from *Rhizomucor miehei* (Novozyme RMIM). However, the product contains only 65% palmitic acid in *sn*2-position causing undesired side effects in infants due to the formation of calcium soaps from palmitic acid present in the *sn*1- or 3-position. In order to obtain higher purities and yields than those achieved by simple acidolysis, we developed an elegant and scalable two-step lipase-catalyzed process *(figure 1)*. First, the triglyceride (i.e., tripalmitin) is subjected to alcoholysis with a lipase from *Rhizopus delemar* and yields highly pure *sn*2-monopalmitin. This strategy suppresses undesired acyl migration, which takes place during hydrolysis or even during acidolysis of triglycerides, leading to the formation of *sn*1- or 3-monopalmitin negatively affecting final product yield and purity. Another advantage is that the *sn*2-monopalmitin can be easily crystallized and hence *sn*2-monopalmitin could be obtained in almost quantitative yield and purity. In the second step, the *sn*2-monopalmitin is esterified by a lipase with two equivalents of oleic acid and yields up to 70% OPO at excellent chemical and regioisomeric purity (96%) (Schmid *et al.*, 1998; Schmid *et al.*, 1999). The same concept was also successfully used to obtain other sTAG (Soumanou *et al.*, 1997; Soumanou *et al.*, 1998) of the CyOCy type or starting from peanut oil. Furthermore, with a *Pseudomonas* lipase up to 80% polyunsaturated fatty acids were present in the *sn*2-position of 2-monoglycerides obtained by alcoholysis from tuna oil and hence this method is very useful to synthesize sTAG with high content of PUFA (Wongsakul *et al.*, 2003).

Later we developed another synthetic route starting from 1,3-diacylglycerides (1,3-DAG), which are available on large-scale as cooking and frying oils or can be obtained from glycerol and fatty acid vinyl esters (Berger *et al.*, 1992). These 1,3-DAGs were then esterified with a lipase exhibiting distinct fatty acid selectivity, i.e. the lipase must not act on the fatty acids present in the *sn*1- and *sn*3-position and solely catalyze the introduction of the second type of fatty acid into the *sn*2-position. We could show that commercial lipase from *Pseudomonas cepacia* (Amano PS) and *Candida antarctica* (CAL-B) allow for the synthesis of sTAG due to their distinct fatty acid specificity (Wongsakul *et al.*, 2004).

Protein engineering to alter the selectivity and stability of lipases

Although many lipases are commercially available, these natural enzymes do not always show the characteristics required for their efficient application in biocatalysis. This includes features such as substrate scope, regio- and stereoselectivity, pH- and temperature profile, but also stability (long term process stability, tolerance of organic solvents etc.). Protein engineering allows to alter these properties and hence represents a key technology in modern biocatalysis (Bornscheuer and Kazlauskas, 2011; Bornscheuer *et al.*, 2012; Kazlauskas and Bornscheuer, 2009; Lutz and Bornscheuer, 2009; Lutz and Bornscheuer, 2012). Methods for protein engineering can be divided into two major concepts: rational protein design or directed (molecular) evolution. Rational protein design requires the 3D-structure of the enzyme of interest (or at least a very good homology model) and with the help of computer programs distinct – usually only a handful – amino acids are then identified and mutated to alternative amino acid residues. These are created in the laboratory and verified experimentally. In contrast, directed evolution is comprised of a random mutagenesis – such as error-prone PCR – resulting in huge mutant libraries (usually $>10^4$-10^7 variants), which are then subjected to either screening or selection to find the desired mutants. Both approaches have their pros and cons, which are well document in literature. Since recently, researchers use also combinations of both approaches, in which residues identified by rational design are subjected to simultaneous saturation mutagenesis resulting in smaller libraries, but at the same time a much higher hit rate can be achieved (Jochens, 2010; Bartsch, 2008).

We have applied protein engineering to improve the stability of a *Rhyzopus oryzae* lipase (ROL) against aldehydes. These compounds are formed in plant oils with a high content of unsaturated fatty acids, which are prone to oxidation. Hence, stable lipases are needed in plant oil processing such as transesterification for margarine production. First, we analyzed the 3D-structure of ROL to identify lysine and histidine residues on the protein surface as these are known to react with aldehydes, e.g. lysine residues will form a Schiff base with aldehydes. Selected amino acid residues were then subjected to saturation mutagenesis replacing the existing amino acid with all other 19 proteinogenic amino acids followed by screening in mirotiterplates (Bertram *et al.*, 2007) to identify more stable ROL-variants. This was done by determining the residual activity of the lipase in the hydrolysis of *p*-nitrophenyl butyrate after incubation of the enzyme with a range of aldehydes. The screening of around 200 mutants per targeted residue – a moderate number for a directed evolution approach – led to the identification of mutant His201Ser with a 60% increased stability. Further combination with mutant Lys168Ile led to a doubled improvement of the stability of ROL while the specific activity was not affected (DiLorenzo *et al.*, 2005, 2007).

In a more recent project, we targeted the fatty acid chain selectivity of lipases. Since medium chain fatty acids (C_6-C_{10}) and their corresponding triglycerides (MCT) provide quick access to energy they represent interesting food additives. As functional oils, they are part of weight loss diets or are used in clinical nutrition. In earlier work Haas *et al.*

applied molecular modeling to identify the structural basis of the acyl chain length specificity of a lipase from *Rhizopus delemar* and predicted variants with significantly shifted chain length specificity (Joerger and Haas, 1994; Klein *et al.*, 1997). Pleiss *et al.* generated variants of the isoenzyme Lip1 of *Candida rugosa* lipase with remarkable differences in the preference of fatty acids with different chain length (Schmitt *et al.*, 2002).

We have used *Candida antarctica* lipase A (CAL-A) as this is the first representative of a new subclass of lipases because of its unique cap domain. The acyl-binding tunnel is mainly formed by this domain. In order to create CAL-A variants with a high specificity for medium chain length acids (C_6–C_{12}), we used rational protein design to block the primary acyl-binding tunnel of CAL-A at position G237 (*figure 2, left*). In order to test this hypothesis experimentally, four different CAL-A mutants (substitutions with alanine, valine, leucine or tyrosine, G237A/V/L/Y) were generated at position G237 and their chain length profile was determined first with *p*-nitrophenyl esters (pNP-esters) and finally also with triglycerides. Whereas wildtype CAL-A shows good activity towards pNP-esters ranging from C_4-C_{18}, already mutant G237A shows a significant reduction of activity towards long chain fatty acids. Especially the three CAL-A mutants G237L/V/Y demonstrated very distinctive chain length profiles and were unable to hydrolyze fatty acids chains longer than C_6. In addition, variants G237V and G237Y showed an up to 3-fold higher activity in the hydrolysis of pNP-butyrate and pNP-hexanoate compared to CAL-A wildtype. Therefore, we could show that by the closing of the junction to the primary acyl-binding tunnel at position G237, CAL-A variants highly specific for medium chain fatty acids could be created (Brundiek *et al.*, 2012).

In another project, we targeted to create a lipase with distinct selectivity for *trans*-fatty acids (TFA), which are present in partially hydrogenated oil and had been identified as an important risk factor for coronary heart disease. Still approximately 5% of the world plant oil production is still subjected to hydrogenation, although alternatives such as fractionation and interesterification have gained more relevance in the production of ''zero *trans*'' edible fats (Destaillats *et al.*, 2009). Until recently, neither chemical nor enzyme catalysts that enable the highly selective removal of TFAs from partially hydrogenated vegetable oils (PHVO) had been described. Our starting point was a publication, in which it was shown that CAL-A exhibited a 15-times higher reaction rate in the esterification of elaidic acid ($C_{18}\Delta9$-*trans* fatty acid) over oleic acid ($C_{18}\Delta9$-*cis* fatty acid) with *n*-butanol (Borgdorf and Warwel, 1999). After we could establish functional expression of CAL-A in *E. coli*, we performed computer modeling based on the 3D-structure of the lipase and inspected all residues comprising the ~30 Å long acyl binding tunnel of CAL-A. 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. These were then subjected to individual saturation mutagenesis and after expression in deep-well plates, we screened about 5,000 clones by an assay with pNP-elaidic acid and pNP-oleic acid esters to identify variants with an improved selectivity for *trans*- over *cis*-fatty acids. Whereas wildtype CAL-A hydrolyzed the elaidic acid-pNP-ester with a 2.5-fold preference in this reaction system, the best variant was 15-fold selective. In the next step, the best mutants were used for the hydrolysis of a PHVO, which is a complex mixture containing over 70 saturated and unsaturated fatty acids with differing chain lengths, numbers and positions of double bonds constituting the triglycerides. The oil used in this study contained 18% *trans* fatty acids with approximately 20 different types of fatty acids. Due to this complex mixture, samples from lipase-catalyzed hydrolysis were analyzed by gas chro-

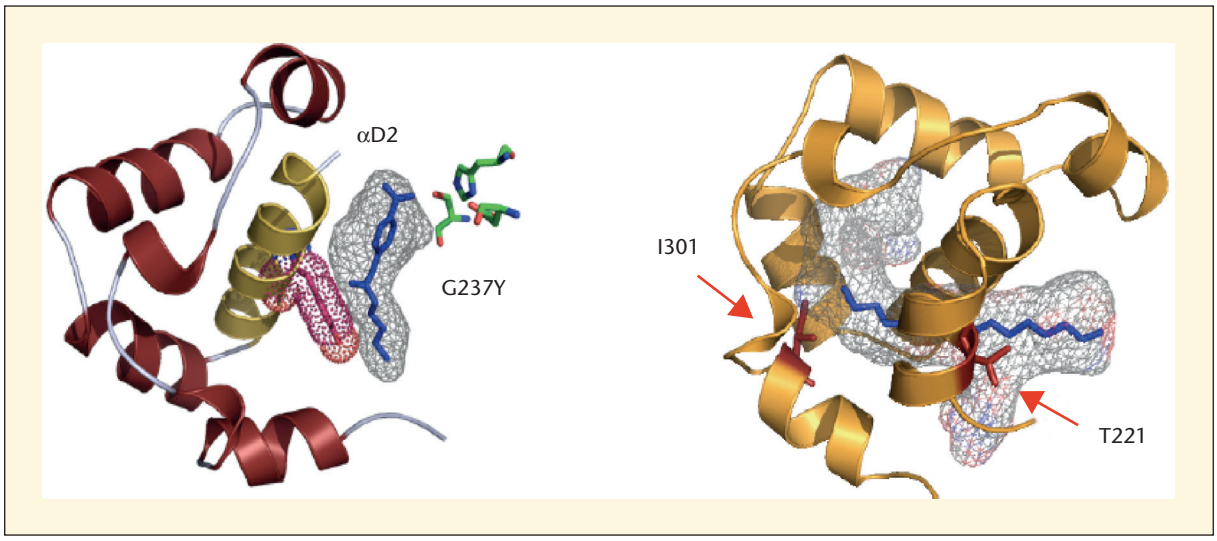

Figure 2. *Left: Acyl binding site of CAL-A mutant G237Y modeled with docked* p*-nitrophenyl caproate to illustrate the position of this medium chain fatty acid in the CAL-A binding tunnel (shown as wireframe). The tyrosine substitution in helix aD2 is shown as red stick, the active site residues are shown in green. Right: Structure of CAL-A indicating positions T221 and I301, which had the most pronounced effect on the* trans*-fatty acid selectivity of this lipase.*

matography. This revealed that the CAL-A mutants T221H and I301H (*figure 2, right*) exhibited excellent selectivity and solely hydrolyzed *trans*- and saturated fatty acids, but no *cis*-fatty acids could be detected and thus remained bound to the glyceride fraction. Hence, these lipase variants do not differentiate between saturated and trans fatty acids, but primarily between those that can adopt linear geometries (saturated and trans-fatty acids) in contrast to cis fatty acids with a kinked structure. Therefore these CAL-A variants are very well suited to remove trans-fatty acids from partially hydrogenated plant oils.

Application of other enzymes in lipid modification

Enzymatic removal of 3-monochloro-1,2-propane diol (3-MCPD)

3-MCPD is a toxic contaminant in processed food well known for about 30 years. More recently, this compound has observed attendance due to its occurrence as fatty acid esters in edible oils and products derived from them (Weisshaar, 2011). In the past few years, considerable efforts were undertaken to either avoid formation of 3-MCPD during oil processing or for its removal by physicochemical treatments. We have developed an alternative enzymatic approach to remove 3-MCPD and its esters from aqueous and biphasic (plant oil/water) systems (Bornscheuer and Hesseler, 2010). For this, 3-MCPD is converted by an enzyme cascade consisting of a halohydrin dehalogenase from *Arthrobacter sp* AD2 and an epoxide hydrolase from *Agrobacterium radiobacter* AD1. The dehalogenase converts 3-MCPD into the corresponding glycidol, which is then quantitatively hydrolyzed by the epoxide hydrolase to the non-toxic glycerol *(figure 3)*. The method not only worked for the removal of 3-MCPD, but in combination with *Candida antarctica* lipase A also the corresponding oleic acid monoester of 3-MCPD could be efficiently converted into glycerol. Moreover, the reaction takes place at low water concentrations of only 5% in the plant oil to facilitate its use on large scale (Bornscheuer and Hesseler, 2010).

Discovery of phospholipase C for enzymatic degumming

Phospholipases are divided into four groups (PLA_1, PLA_2, PLC and PLD) depending on their site of action on the phospholipid molecule. PLA_1 and PLA_2 are used on large-scale for degumming – the removal of phospholipids – of natural fats and oils since decades. Early processes used a mammalian phospholipase from porcine pancreas specific for the *sn*2-position (PLA_2), but this method was later replaced by an enzyme obtained from *Fusarium oxysporum*, which exhibits *sn*1-selectivity (PLA_1). More recently, researchers at Novozymes created a chimeric enzyme by protein engineering from a lipase scaffold and parts of the *Fusarium* enzyme. The action of these two enzymes releases lysophospholipids, which are easily hydrated and therefore allow the reduction of the phospholipid content to < 10 ppm. Another alternative is to use phospholipase C (PLC, EC 3.1.4.3) that specifically hydrolyze the C-O-P bond in phospholipids, yielding *sn*-1,2(2,3)-diacylglycerides and the phosphate residue bearing the corresponding headgroup. This process has the advantage that no oil loss occurs and the removal of the phosphate can be as efficient as with PLA_1 or PLA_2. A PLC-based process has recently been established by the company Verenium (now DSM, San Diego, USA) on large scale. In one project, we also discovered a range of PLC after screening several *Bacillus* sp. strains and could functionally express the PLC in *Bacillus subtilis* (Durban, 2007a) for use in degumming. Biochemical characterization of PLC requires methods for the reliable determination of their activity. For this we developed an assay in which the phosphate residue released by the PLC is cleaved with an alkaline phosphatase. The phosphate formed is then extracted with *n*-butanol and quantified as a phosphomolybate complex. The applicability of this method was demonstrated for a concentration range from 10 nM to 10 mM for a range of phospholipids bearing different headgroups in an aqueous and a two-phase system and could be used reliably even with crude non-purified PLC (Durban, 2007b).

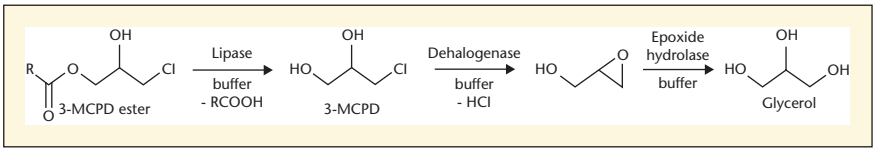

Figure 3. *A combination of lipase, dehalogenase and epoxide hydrolase can be used for the efficient conversion of the toxic contaminant 3-monochloropropanediol (3-MCPD) or its fatty acid ester present in processed plant oils to the harmless glycerol.*

Conclusions

The examples summarized in this contribution demonstrate that enzymes are very useful catalysts for lipid modification due to their excellent regio- and chemoselectivities. Furthermore, the outcome of a biocatalytic reaction can be optimized by careful control of the reaction conditions – as shown for the two-step synthesis of structured triglycerides – or the enzyme can be tailor-designed by methods of protein engineering.

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