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Topical Issue

Enzymes for industrial applications

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Abstract – Protéus is a biotechnology company specializing in the discovery, engineering and production of enzymes for industrial applications, as well as in the development of innovative bioprocesses involving these enzymes. Protéus is a subsidiary of the PCAS Group, actor in fine chemicals and specialty products and producer of high-value complex molecules. Enzymes allow considering unique functionalizations that are difficult to achieve by conventional chemical means. Examples involving the screening of our ready to use toolbox of lipases, the engineering of the well-known lipase CalB and the specific modifications of lipids will be presented.

Keywords: lipids / lipases / protein engineering / bioprocesses / biocatalysis

Résumé – Développement d'enzymes pour des applications industrielles. Protéus est une société de biotechnologie spécialisée dans la découverte, l'ingénierie et la production d'enzymes pour des applications industrielles, ainsi que dans le développement de bioprocédés innovants mettant en œuvre ces enzymes. Protéus est une filiale du groupe PCAS actif dans le développement, la fabrication et la commercialisation de produits de chimie fine et de spécialités. Les enzymes permettent de produire de nouvelles molécules dans un contexte de chimie verte (économie d'atomes, conditions douces de mise en œuvre, amélioration de la sélectivité, réduction de la toxicité) et d'envisager des fonctionnalisations uniques qui sont difficiles à obtenir par des moyens chimiques classiques. Des exemples de réalisation seront présentés avec notamment le criblage de notre *toolbox* enzymatique, l'ingénierie de la lipase issue de *Candida antarctica* et la fonctionnalisation de liaisons CH non activées.

Mots clés : lipides / lipases / ingénierie de protéines / bioprocédés / biocatalyse

1 Introduction

There are two ways Protéus develops new proteins for industrial application: mining the natural biodiversity through a collection of more than 8000 microorganisms (bacteria, archaea, fungi and algae) and bioengineering through technologies like gene DNA shuffling or random mutagenesis. Protéus collection is constituted of microorganisms that are proprietary or acquired through partnerships with industries and academia. These microorganisms have been isolated from a great variety of natural environments. Also, Protéus has patented two technologies on directed evolution. The first one, L-Shuffling™, allows the recombination of a pool of parental genes by ligation, and the second one, Evosight™, is based on a mathematical model for determining the optimal mutational load to apply in random mutagenesis.

Otherwise, recent optimization studies based on design of experiments (DoE) led to a more than 4-fold increase in the yield of an enzymatic clean process for manufacturing a major ingredient for flavors and fragrances. DoE are mainly used to develop robust processes for application under difficult conditions. High viscosity, foam, low solubility of substrate(s) and/or product(s), phase separation or emulsion formation are some of the typical challenges that Protéus can help to overcome.

In a first step, a brief non-exhaustive description of the enzymes acting on lipids and fatty acids will be reviewed. Then, specific examples will be presented concerning the screening of our collection of lipases and esterases, the engineering of the widely-used lipase CalB, and the specific modifications of lipids.

2 Enzymes for lipids/fatty acids structural modifications

Several enzymes have been described for their activity toward lipids or fatty acids. These enzymes can be used either for triglyceride cleavages, hydrolysis, esterification or transesterification of fatty acids/esters, or for specific functionalizations of fatty acids/esters (hydroxylation, epoxidation).

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3 Lipases

Lipases are ubiquitous. They are produced by a broad variety of organisms including animals, plants and micro-organisms. They have been used in a wide-variety of applications in the fields of pharmacy, fine chemistry, general health, food, cosmetics, environment and energy. Lipases are serine hydrolases defined as triacylglycerol acylhydrolases (E. C. 3.1.1.3). Lipases are involved not only in the hydrolysis of long chain aliphatic acid esters but also in esterification/transesterification reactions. Lipases also catalyze a large variety of synthetic reactions such as thio-esterification and amidation. Esterases are also very useful enzymes for the same type of biotransformation.

Many lipases have industrially relevant properties such as chain length specificity, regiospecificity and chiral selectivity. Lipases are often used for preparing enantiopure compounds from racemic mixtures, diastereoisomeric mixtures or prochiral molecules. Globally, these specificities depend on the shape of the binding site and the nature of the amino acids composing the binding site. For the enantio-selectivity, the specificity also depends on the structure of the substrate (flexible/rigid). Physicochemical factors such as temperature and solvent can modify the enantio-preference of an enzyme. Synthetic reactions involving lipases may be conducted in a medium with low thermodynamic water activity, consisting for instance of solvent–free or organic solvent systems.

4 P450 monooxygenases

With NAD(P)H as a cofactor, P450s catalyze the insertion of one oxygen atom from molecular oxygen into an organic substrate through electron transfer. Fatty acid-hydroxylation P450s are classified into carboxyl-terminal hydroxylases (α-hydroxylases) and terminal or subterminal hydroxylases (ω-hydroxylases) (Van Bogaert *et al.*, 2011).

5 Hydratases

Hydratases (EC 4.2.1.53) catalyze the regio-specific, irreversible addition of a hydrogen atom and a hydroxy group from water to the carbon–carbon cis-double bond of unsaturated fatty acids at the C9 and C10 positions, respectively, to make 10-hydroxy fatty acids (Joo *et al.*, 2012).

6 Hydroxylases

12-Hydroxylases (EC 1.14.13.26) convert oleic acid to ricinoleic acid by catalyzing the site-specific hydroxylation at the 12-position of oleic acid in the presence of NADH using O_2.

7 Lipoxygenases

Lipoxygenases (EC 1.13.11), a family of dioxygenases, catalyze the insertion of molecular oxygen into polyunsaturated fatty acids (PUFAs) containing one or more cis, cis-pentadiene units to produce the corresponding hydroperoxy fatty acids, which are thereafter reduced to hydroxy fatty acids (Brash, 1999; Ivanov *et al.*, 2012).

Finally, as described by Tiran *et al.* (2008), P450 monooxygenases, diiron-center oxygenases, lipoxygenases, peroxygenases, and hydrolases are enzyme classes involved in free fatty acid epoxidation.

8 Protéus's toolbox

Over the past 18 years, Protéus has assembled a toolbox of enzymes ready to be screened. This toolbox includes alcohol dehydrogenases, monooxygenases, laccases, lipases, esterases, amidases, proteases, dehalogenases, epoxyde hydrolases, nitrilases, nitrile hydratases and aldolases. Among the lipases and esterases within this toolbox, there are about 40 available. Lipases are characterized either by a high enantioselectivity or a high tolerance toward a large range of pH and temperatures. Some of them are also particularly active toward secondary alcohols with performances far superior to commercial enzymes. For instance, a screening for thermostable esterases has been done in partnership with Goldschmidt (now Degussa) (Ravot *et al.*, 2004).

The use of esterases that are active and stable at high temperature could open biocatalytic routes to novel substances that are competitive to conventional chemical routes. For example, organic solvents are often needed to solubilize solid lipid substrates and products. However, if the reaction can be performed at higher temperature at which the substrates or products become liquid, there is no need to add (and remove at the end) organic solvents. Therefore, the reaction can be performed at lower cost (by reducing the product recovery process) in an environmental friendly process (by reducing harsh chemical conditions) (Lagarde *et al.*, 2002).

For example, with Degussa, Protéus screened various thermophilic microorganisms selected within the archaea and the bacteria domains. We identified a new extremely thermostable esterase having a broad substrate specificity from a hyperthermophilic archeon belonging to the genus *Pyrococcus* isolated from deep sea hydrothermal vent. This thermostable enzyme is active for catalysing hydrolysis, esterification, transesterification or amide formation over a wide temperature range of 40 °C up to 110 °C. To produce this esterase, an efficient, simple, and reliable production process using *Escherichia coli* was developed. This process was robust and further upscaling to industrial scale was achieved without any problem, making the use of this esterase economically competitive for the industrial production of fine chemicals.

9 Lipase engineering

When an enzyme that fully fits within required specifications is not available, an enzyme can be engineered from a wild type enzyme by modifying its genomic sequence using a single gene or a pool of parental genes from various species. In the following example, we set out to improve the specificity of the enzyme CalB in the transesterification between a fatty ester and a triol, of which only one of the three alcohol functions is to be selectively converted. Because the three alcohol functions of the triol are distinguishable due to their environment, two primary alcohols, one situated on a linear part of the molecule, the other situated in substituted region, and a secondary alcohol, there is a potential to form

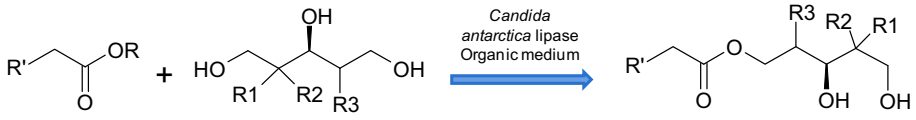

Fig. 1. Esterification catalyzed by *Candida antarctica* lipase.

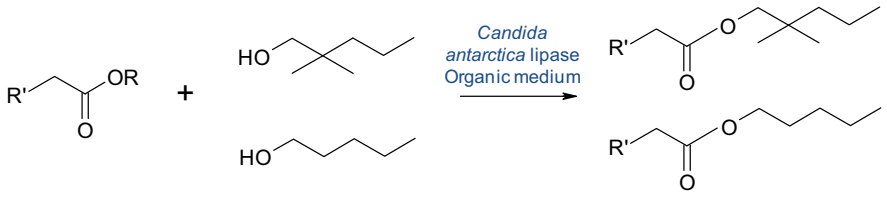

Fig. 2. Model reaction developed by Protéus.

three distinct esters. However, the primary alcohol situated on the linear part of the molecule is the intended target (Fig. 1). Also, the ester substrate contains an impurity two carbons short.

As a consequence, in addition to the desired product, several undesired products were obtained with the native CalB. The objective was to improve the enzyme CalB to increase the proportion of the target compound compared to co-products. An improvement of the reaction rate was a secondary objective.

A library of mutants was constructed using Protéus Evosight™ proprietary technology. The mutants were expressed in *E. coli* and freeze-dried.

In order to speed up the high throughput screening process, two model alcohol substrates were used: the first one mimicking the linear primary alcohol and the second one mimicking the substituted primary alcohol (Fig. 2). Both these alcohols were mixed in equal amounts with the fatty ester substrate and the freeze-dried mutant enzymes in pure organic solvent. The products of the reaction were analyzed using Gas Chromatography coupled with Flame Ionization Detector. By comparing the selectivity results obtained between this model reaction and the targeted reaction, we confirmed that the results of the model reaction are correlated with the ones of the target reaction. Therefore, the ratio of the activities of the mutants on each model substrates (linear/substituted) was the criteria for selectioning the optimized mutants. Mutants with the highest ratio (activity on linear substrate/activity on substituted substrate) were selected for further analyses.

In a first step, seven Polymerase Chain Reaction error-prone libraries were constructed (Fig. 3), each having an average mutational load within the window of 2.1 mutations per gene to 11.2 mutations per gene. The fraction of active mutants in each library was used to determine the optimal mutation load (OML) using a proprietary algorithm. The OML was found to be 3.6 mutations per gene. Therefore, the library having the mutational load closest to the OML was selected and its 14 500 clones were screened.

The best mutants were selected from the high-throughput screening and immobilized on a suitable resin in order to be tested on the target reaction for confirmation and final selection. Their sequences were determined. Nine mutants were identified, two mutants having a strong improvement in

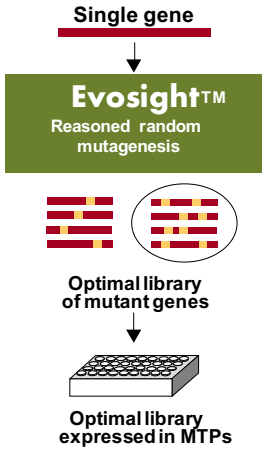

Fig. 3. General scheme of random mutagenesis strategy.

the selectivity towards the desired alcohol function of the triol, five mutants having a reduced selectivity towards the impurity, and two mutants having improved conversion rates. Very interestingly, the locations of the mutations responsible for the improvement of each criterion were different. Therefore, in a following step, the objective will be to combine the positive mutations from each criterion on the same gene using Protéus's L-Shuffling™ technology.

10 Fatty acids/lipids enzymatic functionalization

Other enzymes involved in lipids or fatty acids functionalizations have also been studied at Protéus. The introduction of functional groups, such as hydroxy or epoxy, or the oxidation of non-activated carbon is still a challenge even by enzymatic routes; however, progress has been observed over the last 10 years.

For instance, the specificity of the hydroxylation position has been studied by Protéus and Firmenich by engineering cytochrome P450 BM3 of *Bacillus megaterium* (Brühlmann *et al.*, 2014). The ability of P450 monooxygenases to introduce atmospheric oxygen into non-activated carbon hydrogen

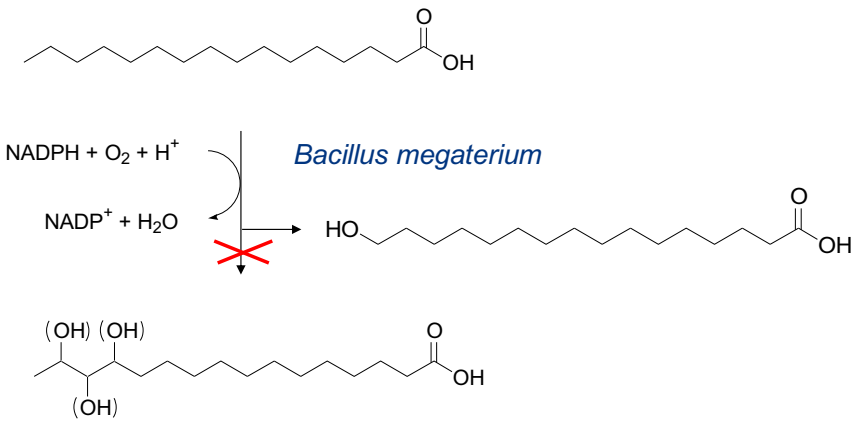

Fig. 4. Reaction targeted through the engineering of cytochrome P450 BM3 from *Bacillus megaterium*.

bonds, often at excellent chemo-, regio-, and stereoselectivity, is a remarkable feature of these enzymes (Meunier *et al.,* 2004; O'Reilly *et al.,* 2011). One of the best studied P450s is the soluble, self-sufficient fatty acid hydroxylase CYP102A1 of *B. megaterium* (BM3). This enzyme is best known for catalyzing the subterminal hydroxylation of long chain fatty acids in a regio-dependent, stereoselective manner (Boddupalli *et al.,* 1992; Miura and Fulco, 1975; Truan *et al.,* 1999).

Terminal hydroxy-fatty acids can serve as building blocks for the preparation of lactones (flavors and fragrances), or for polyesters (coatings, packaging etc.), among others. In partnership with Firmenich, we decided to modify the regioselectivity of the P450 monooxygenase from BM3 toward the formation of terminal hydroxy palmitic acid (Fig. 4). Iterative cycles of random and targeted mutageneses were successfully applied to evolve variants of CYP102A1 towards high selectivity for the oxidation of the terminal position of palmitic acid. These variants acquired obvious as well as many non-obvious mutations. Substitutions at sites F87 and V328, in proximity to the bound substrate, were identified in the first round of random mutagenesis and were retained in subsequent generations. The substitution I263F was identified as a strong regioselectivity determinant only in the fourth round of mutagenesis and is believed to act in cooperation with F87I and A328V. However, additional alterations at several sites more distant from the bound substrate showed surprisingly strong contributions to the improved terminal oxidation selectivity. Though the improved regioselectivity had to be traded for a decoupling of the cofactor NADPH, there is a good chance to regain coupling as demonstrated by Fasan *et al.* (2007). Nevertheless, we provided proof that the bacterial cytochrome P450 BM3 of *B. megaterium* shows enough structural plasticity for its evolution into a terminal fatty acid hydroxylase, which is of interest for a bacterial production platform of omega-hydroxy fatty acids.

Again in partnership with Firmenich, Protéus optimized a 13-hydroperoxide lyase for the industrial production of saturated and unsaturated C6-aldehydes and their derivatives from fatty acids (Brühlmann *et al.*, 2013). In this case, two products, a residual shorter oxidized fatty acid and a C6-aldehyde, can be of industrial interest (Fig. 5). Firmenich was interested in the production of a group of molecules known as "green notes" offering appreciated green, grassy, fatty and fruity odors. This group of molecules is usually produced by plants and find applications in fragrances and flavors. In nature, herbivore attack or mechanical wounding of higher plants initiates the synthesis of C6-aldehydes *via* the well known 13-lipoxygenase pathway.

Because of the very low concentrations of these molecules in plants, their isolation *via* steam distillation or extraction is normally neither an economical, nor a sustainable option. The fragrance and flavor industry has developed synthetic as well as biosynthetic routes to produce C6-aldehydes and derivatives. For example, a "one-pot" process has been exploited based on the 13-lipoxygenase pathway using hydrolyzed seed oils as substrates and homogenates prepared from soybeans and guava as sources of the 13-lipoxygenase and of the 13-hydroperoxide lyase (13-HPL), respectively (Muller *et al.*, 1995; Whitehead *et al.*, 1995). In a first step, unsaturated fatty acids homogenized in aqueous solution are oxidized with oxygen in a regio- and stereoselective manner using a 13-lipoxygenase. The 13-lipoxygenase can be added in form of ground soy beans, which are a rich source of this enzyme. In a second step, the formed fatty acid hydroperoxides are cleaved *in situ* by the addition of a 13-hydroperoxide lyase in form of homogenized fruits such as guava, for example. 13-Hydroperoxide lyases belong to the CYP74 family and catalyze the homolytic isomerization of fatty acid hydroperoxides into short lived hemiacetals, which decompose into C6-aldehydes and the corresponding C12-oxo-acid moieties (Grechkin and Hamberg, 2004). *In situ* isomerization or the use of baker's yeast for the reduction of the aldehydes into the corresponding alcohols has optionally been applied. However, low productivity, fluctuating raw material costs and qualities have contributed to a non-economically viable process. The productivity limiting step for producing C6-aldehydes in such a process is the cleavage of the fatty acid hydroperoxides. This is due to the instability and low concentration of the 13-HPL in plant homogenates and its rapid inactivation during the cleavage reaction. The need for a better source of the 13-hydroperoxide lyase motivated efforts to identify and clone the gene of the 13-HPL of guava (Tijet *et al.*, 2000). Although the 13-HPL was well expressed in a heterologous host such as *E. coli*, it was found by the same authors that most of the recombinant enzyme was non-functional due to protein misfolding (inclusion bodies).

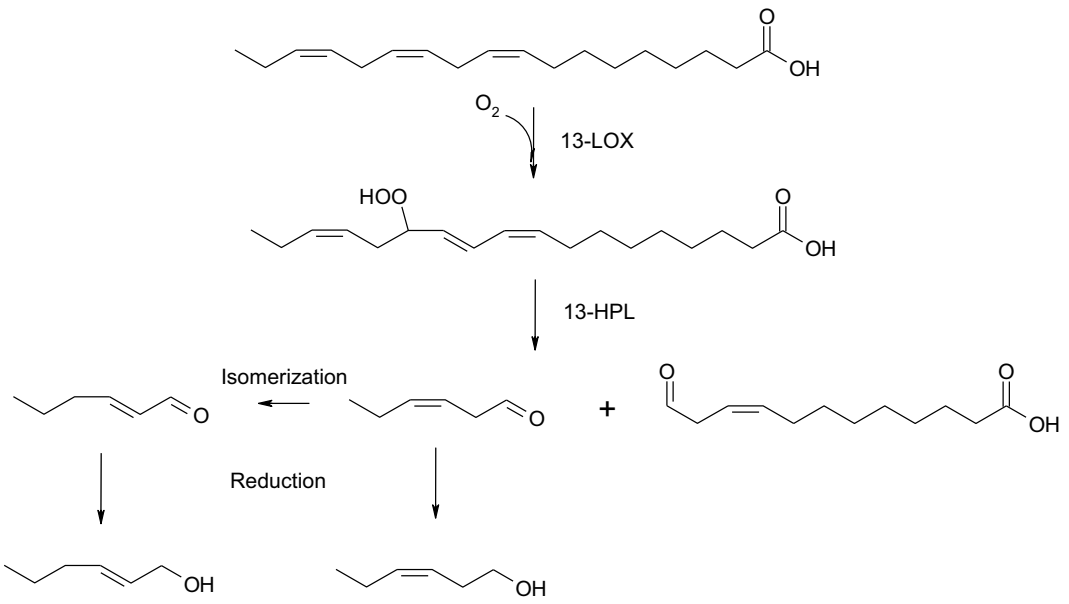

Fig. 5. Linolenic acid submitted to 13-LOX and 13-HPL provides hexanal and *n*-hexanol as products. 13-LOX, 13-lipoxygenase, 13-HPL, 13-hydroperoxide lyase.

At Protéus, a protein engineering strategy based on directed evolution was applied. Four rounds of gene shuffling and random mutagenesis improved the functional expression in *E. coli* and led to a 15-fold higher productivity. A structure-based sequence alignment of the best variant with the recently solved allene oxide synthase of *Arabidopsis thaliana* showed that most amino acid alterations occurred on the surface of the protein, distant from the active site. This study demonstrated the power of directed evolution for improving a complex trait such as the total turnover number of a cytochrome P450, a critical parameter for process performance that is difficult to predict even with good structural information at hand.

11 Conclusion

Lipids and fatty acids are the most abundant renewable raw materials of the chemical and food industries. Their functional modification either by classical chemistry or by biocatalysis is of great importance. As demonstrated above, functionalization of non-activated carbon has been achieved with success with monooxygenases. Also, optimization of a lipase has allowed a great improvement in its selectivity (decrease in side-products formation) and activity.

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