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Protein engineering for development of new hydrolytic biocatalysts

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Hydrolytic enzymes play important roles as biocatalysts in chemical synthesis. The chemical versatility and structurally sturdy features of *Candida antarctica* lipase B has placed this enzyme as a common utensil in the synthetic tool-box. In addition to catalyzing acyl transfer reactions, a number of promiscuous activities have been described recently. Some of these new enzyme activities have been amplified by mutagenesis. Epoxide hydrolases are of interest due to their potential as catalysts in asymmetric synthesis. This current update discusses recent development in the engineering of lipases and epoxide hydrolases aiming to generate new biocatalysts with refined features as compared to the wild-type enzymes. Reported progress in improvements in reaction atom economy from dynamic kinetic resolution or enantioconvergence is also included.

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Introduction

The use of enzymes in organic synthesis is referred to as biocatalysis [(see Ref. 1 for a recent topic update]). There are clear advantages of applying enzymes in synthetic protocols. Enzymes are often unchallenged as catalysts thereby improving synthesis efficiency, economy, and provide a path towards more sustainable manufacturing of chemicals. One can tick off several of the points listed in the Principles for Green Chemistry [2] if enzymes were to replace traditional organochemical and metalloorganic catalysts at a wider scale.

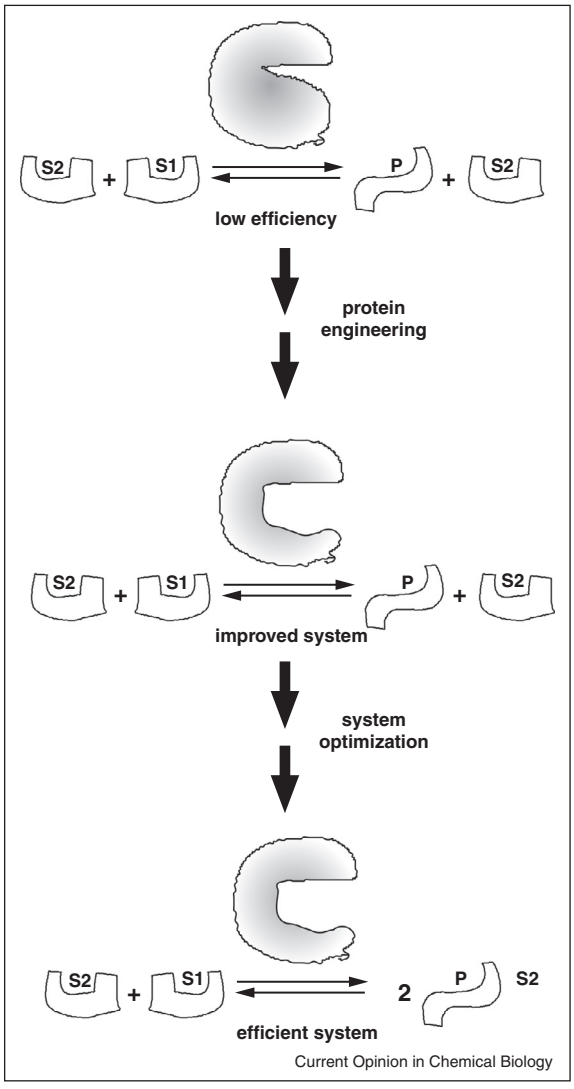
The sources of enzymes that are utilized as biocatalyst are diverse and range from isolates from classical model organisms such as *Escherichia coli* or bakers yeast to extremophilic microbes and metagenomes of particular microbiological societies [3,4••]. Additionally, contemporary methodologies for protein engineering have facilitated introduction of desired modifications to existing enzymes [5••]. This current update describes the recent progress of how different levels of protein (re-)engineering together with other optimizations, such as dynamic kinetic resolutions, in two important classes of hydrolytic enzymes, lipases and epoxide hydrolases, have contributed to the development of new useful biocatalysts (Figure 1).

The biocatalysis community has been successful in isolating enzymes facilitating production of an increasing range of desired products but the majority of applied reaction types have been limited, with hydrolysis and acyl transfer reactions being the most widely adopted. Enzyme (lipase) catalyzed transacetylations are common practice today also within the organic chemistry community. However, the underlying structure–activity related mechanisms that cause the desired product formation have been, if not neglected, of lower priority. Enzymologists, on the other hand, have produced structure–activity data on a range of important model enzymes that are often of low applied value as biocatalysts. This has led to a knowledge gap between these disciplines, one more applied and the other fundamental. An attempt to address this issue and contribute to bridging the gap was the STRENDA initiative [7] which described guidelines for presentation of functional data in isolated biocatalysts.

Lipases

Lipases A and B from *Candida antarctica* have historically been the most applied biocatalysts and for many non-biochemists the quintessential representatives of enzymes in general. The success of lipase B (CALB) as biocatalyst can be considered as something of a mixed blessing for the field. This enzyme displays, on one hand, many desirable properties, both functionally and physicochemically. It is remarkably stable in various organic solvents which renders it perfect as catalyst of transacylation reactions [8] and also allows for solubilization of hydrophobic reactants and products. CALB is also relatively thermostable which further affects reaction rates favorably. These properties, especially the stability in non-polar solvents are, on the other hand, quite unique and enzymes in general do not accept such an environment. (Hence, CALB is not a good representative of enzymes in general and the realization of the synthetic chemist that most enzymes denature and lose catalytic activity under such conditions can be off-putting when

Figure 1



Stepwise improvement of an enzyme catalyzed reaction. A non-natural reaction catalyzed by an enzyme with low catalytic efficiency for the reaction at hand may be improved by protein engineering. The engineering may target single or a few, predefined residues, or be more extensive applying directed evolution to achieve desired enzyme properties. In the given example, one mirror image (S1) of a racemic starting material is converted into the desired product (P) while the other enantiomer (S2) of the starting material is not utilized. In the optimized system both enantiomers are converted into desired product. This can be achieved through dynamic kinetic resolution or enantioconvergence [6•].

designing a synthetic strategy and biocatalytic approaches may be excluded.)

CALB has been primarily applied in catalysis of transesterification reactions but also displays promiscuous activities. Various protein engineering efforts in recent years have targeted improved catalytic efficiencies and altered substrate and stereoselectivities. A noteworthy contribution was engineering by circular permutation [9] where one enzyme variant (dubbed cp283) exhibited improved hydrolysis activity with esters **1a–1c** (Table 1) [10]. The reason for the activity increase was traced to a structural rearrangement of a loop that allowed for faster substrate entrance and product exit in the mutant [11]. In a CALB-catalyzed promiscuous reaction, an S105A point mutant (S105 is the catalytic nucleophile otherwise required for acyl transfer reactions) catalyzed C–C bond formation in Michael addition reactions (Scheme 1) [12] as well as hydrogen peroxide afforded epoxidation of α,β-unsaturated substrates [13]. Further, in a combined bioinformatics and computational study mapping amino acid residues required for hydrolytic activity in structurally related amidases and lipases of the α/β-hydrolase superfamily, candidate residues responsible for amidase activity were identified. Although catalytic groups were superimposable between the compared amidases and lipases, structural differences in neighboring loops were observed. When corresponding mutations were introduced into CALB, enzyme variants displaying up to 11-fold improvement in hydrolytic activity with **2** were identified [14]. In another study, also targeting to improve upon the miniscule amidase activity of wild-type CALB, a substrate-contributed hydrogen bond proposed to lower the activation barrier of cleavage of the scissile C–N bond was used as role model for protein engineering [15]. Residue I189 was mutated into residues potentially capable of contributing hydrogen bonds, and might thereby facilitate amidase activity [16]. Both the I189Q and I189N variants did indeed exhibit increased preferences for hydrolysis of **3** as compared to the corresponding ester analog.

Kinetic resolution of accepted substrates is an often applied approach to yield production of enriched enantiomeric excess of products. A drawback with this strategy is the maximum 50% conversion of starting material into desired product. A more desirable strategy would result in full conversion but require enantioconvergence or dynamic kinetic resolution. The latter has been reported for a CALB-mutant (W104A) that catalyzed transacetylation of phenyl substituted *sec*-alcohols. The mutation allows for larger substrates than ethyl-substituted derivatives to be accepted and also changes the enzyme's stereoselectivity to prefer (*S*)-enantiomers. A rhutenium-based metalloorganic catalyst was included to catalyze racemization of remaining alcohol thereby refilling the depleted (*S*)-enantiomer of the reactant [17•]. The same strategy has been described for chemoenzymatic synthesis of cyclic ketones [18].

CALB displays poor activity with chiral α-substituted esters, some of which are precursors for synthesis of, for example, ibuprofen derivatives. Reetz and co-workers conducted ISM-driven directed evolution selecting for enzyme variants with activity and stereoselectivity in hydrolysis either enantiomer of **4** [19••]. A number of