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Biosynthesis of the non-canonical amino acid piperazic acid by an enzyme cascade

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PURPOSE OF THE ABSTRACT

Nature provides a large repertoire of non-ribosomal peptides with potent properties for various applications. The underlying bioactivity is especially interesting for the biosynthesis of drugs and related compounds. Respective (depsi)peptides are derived from proteogenic and non-canonical amino acids as well as carboxyclic acids and various combinations thereof. Here, we became intrigued by the biosynthetic machinery of some bacteria to form natural products such as metallophores and antimicrobial agents comprising N-N bonds.[1] The required precursors can be obtained from central metabolic pathways. Interestingly, some bacteria can N-hydroxylate amino acids selectively, such as L-ornithine towards N5-hydroxy-L-ornithine which can be further converted to L-piperazic acid. The latter represents one of those potent non-canonical amino acids. The Actinobacterium Kutzneria sp. 744 encodes the genetic information for the enzymes leading to L-piperazic acid, namely the flavoprotein N-hydroxylase (NMO) and piperazate synthase (PipS).[2] Especially, the latter enzyme is not well described with respect to biochemical properties nor biotechnological application.

A phylogenetic investigation revealed that those enzymes can frequently be identified from Actinobacteria while only one was studied in somewhat detail up to now. This enzyme from Kutzneria sp. 744 (also designated KtzT) was reported to be a heme-protein with a narrow substrate scope. It is part of the biosynthetic machinery towards kutzneride compounds which are active against fungal and bacterial strains.[3] To date, no detailed data on enzyme production, heme-loading, enzyme kinetics, stability, structural information etc. were available. Hence, we cloned and produced the enzyme, reaching mediocre yields (0.4 mg protein per L broth with a heme loading of about 12%) in first attempts. A series of experiments to improve gene expression led to an almost 40-fold improved production of heme loaded and active catalyst. Another problem studying this enzyme was the supply of N-OH comprising substrates. This could be solved by our expertise on N-hydroxylating enzymes.[4,5] Here, we employed the NMOs from Thermocrispum agreste (TheA) to produce N5-hydroxy-L-ornithine or from Gordonia rubripertincta (GorA) to provide other N-OH molecules in situ. As this enzyme class is NADPH-dependent, we had to recycle the liberated $NADP^+$. This was achieved with an FDH variant from Candida boidinii. All in all, we optimized the cascade and were able to use it to study KtzT and related PipS enzymes. Activity was followed by an HILIC-MS/MS approach which allowed to separate substrates and products and to detect compounds by tandem mass spectrometry. For the first time, we were able to demonstrate that the here employed NMOs can produce a series of N-hydroxylated intermediates of which some can be used by PipS.

Our studies were corroborated by iterative bioinformatics approach comprising ligand docking and refinement by molecular dynamics simulations to predict heme positioning and substrate binding in KtzT. This resulted in a trustworthy model of KtzT containing a docked heme and substrate. This information was then employed to conduct mutagenesis experiments as well as to explain substrate promiscuity of this enzyme family for the first time.

In conclusion, we found the family of PipS enzymes is very promising to produce various N-N containing molecules,

especially in artificial enzyme cascades, where further optimization might lead to interesting future applications.

FIGURES

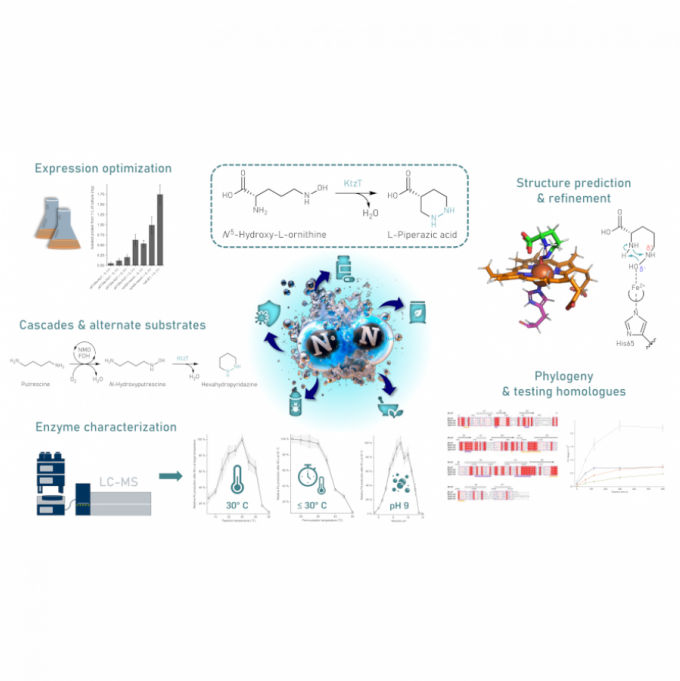

FIGURE 1

KtzT

A biochemical study of KtzT including expression optimization, assay development, activity determinants and structural probing.

FIGURE 2

KEYWORDS

flavoprotein monooxygenase | N-N bond formation | natural product | gene expression

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