Zürcher Hochschule
für Angewandte Wissenschaften

Life Sciences und
Facility Management

ICBT Institut für
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Microbial epimerases for discovery of novel peptide therapeutics

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1. Background

Recent years have witnessed the advent of D-peptides as a new class of peptide therapeutics due to their resistance against enzymatic degradation and prolonged plasma half-life.[1] In Nature, non-ribosomal peptide synthetases (NRPSs) have long been assumed to be solely responsible for the biosynthesis of D-peptides.[2] However, recent reports suggest that the enzyme superfamily of radical *S*-adenosyl-L-methionine (rSAM) epimerases can convert ribosomally-synthesized, all L-peptides into their D-amino acid containing counterparts (Figure 1a).[3-5] Due to the substrate promiscuity of the enzymes, it is our interest to adapt them for modification of natural peptide therapeutics (Figure 1b).

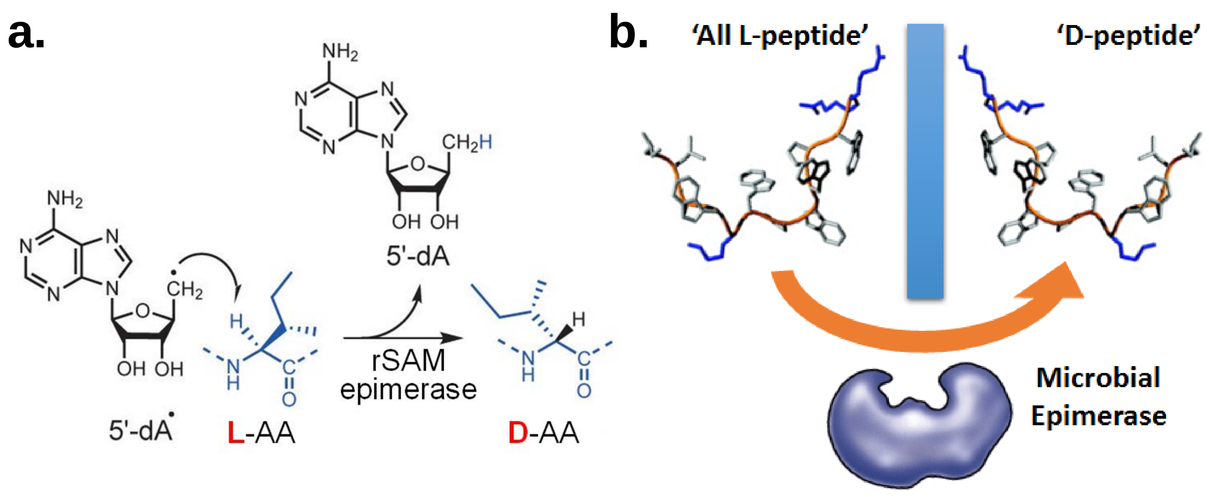

Figure 1. (**a**) Schematic representation of rSAM epimerase catalysis and (**b**) epimerization of 'all L-peptide' to 'D-containing peptide' mediated by microbial epimerase.

Epimerizations reported so far mainly rely on *in vivo* system, i.e., co-expression of the epimerase and the peptide substrate followed by laborious downstream purification processes (HTP not possible).[3-5] Although *in vitro* epimerization has proven challenging due to oxygen sensitive nature of the enzymes, it enables isolation of peptide products by simple separation techniques and allows for any combination of epimerase and accepted substrate peptide in a systematic fashion. Here we demonstrate *in vitro* epimerization using a model epimerase/peptide pair.

2. Material & Methods

We chose an epimerase/substrate peptide pair OspA/OspD from *cyanobacterium Oscillatoria sp*. PCC6506 in our proof-of-principle study.[4] Genes encoding the epimerase OspD (sq.ID: WP_007357380) and substrate OspA (sq.ID: WP_007357382) were ligated into pET28b vectors. To isolate the core peptide after epimerization, a Factor Xa (FXa) site was engineered into the leader-core interface of OspA (Figure 2a). Both proteins were produced in *E.coli* with good yield (~100 mg/L). To maximize the cofactor loading, OspD was anaerobically reconstituted by addition of Na_2S and $Fe(II)SO_4$ in the presence of reductants DTT and $Na_2S_2O_4$. Epimerization reactions were performed in an anaerobic glove box. After reaction, each sample was subjected to desalting and FXa-digestion (to isolate core peptide), followed by LC-MS analysis (Figure 2b).

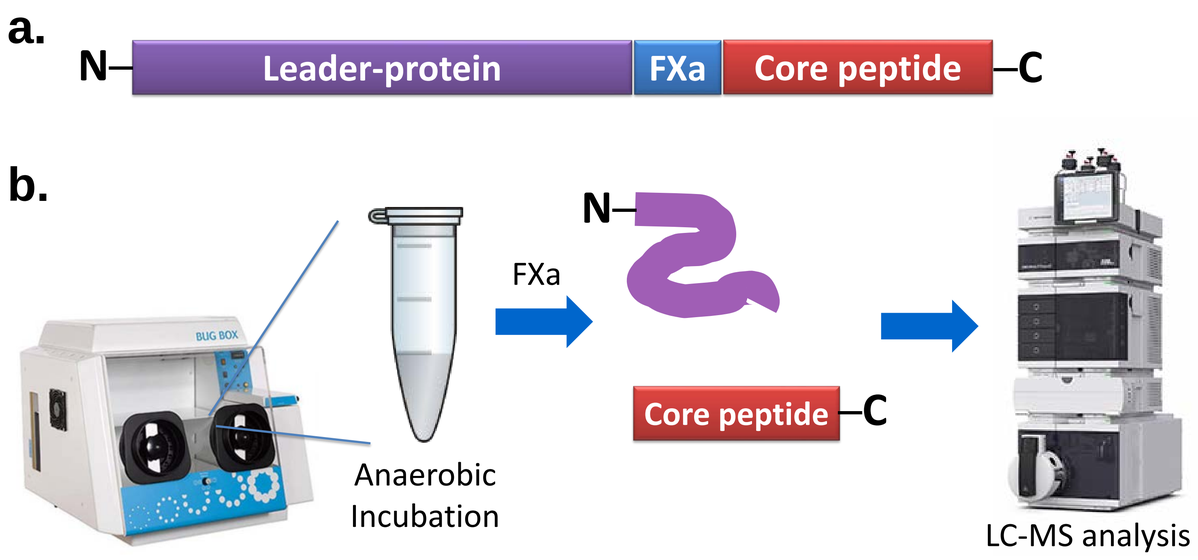

Figure 2. (**a**) Architecture of OspA construct used in this study and (**b**) schematic representation of *in vitro* epimerization and analysis workflow.

3. Results & Discussion

Enzyme characterization

The purified OspD epimerase shows slightly yellow-brownish color but anaerobic reconstitution gave rise to distinctive spectroscopic signature at ~450 and 600 nm (Figure 3a). Using an extinction coefficient,[6] we calculated the iron-sulfer occupancy to be >20%. Structural modeling using iTasser suggested an α-helical bundle structure with the cofactor binding site located near the protein surface (Figure 3b).

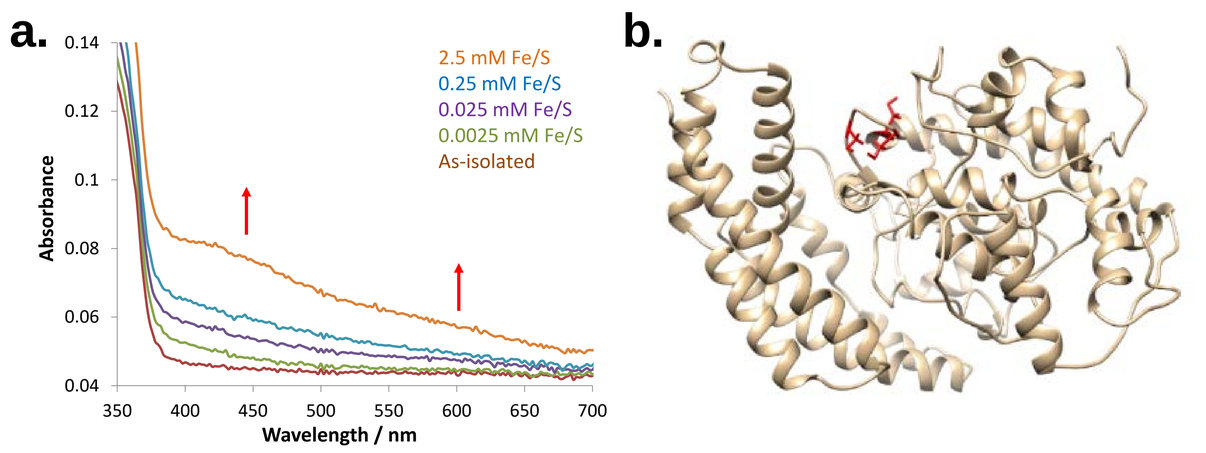

Figure 3. (**a**) UV-vis spectra of OspD reconstituted with various Fe/S concentrations and (**b**) homology model of OspD epimerase built by i-Tasser.

in vitro epimerase activity assay

To examine the activity of OspD, the reconstituted enzyme was incubated with substrate OspA under anaerobic condition. LC-MS analysis of the core peptide show conversion into new species (at least two), consistent with singly and doubly epimerized products, respectively (Figure 4a and b).[4] Optimization of reaction conditions afforded a single species (doubly epimerized products) in 3 hours at room temperature (Figure 4a). When reaction was performed in deuterated buffer, we monitored a mass increase of 2 Da (data not shown),[5,7] confirming the 'radical mechanism' in which hydrogen abstraction by rSAM and subsequent protonation by an exchangeable proton occurs (Figure 4c).

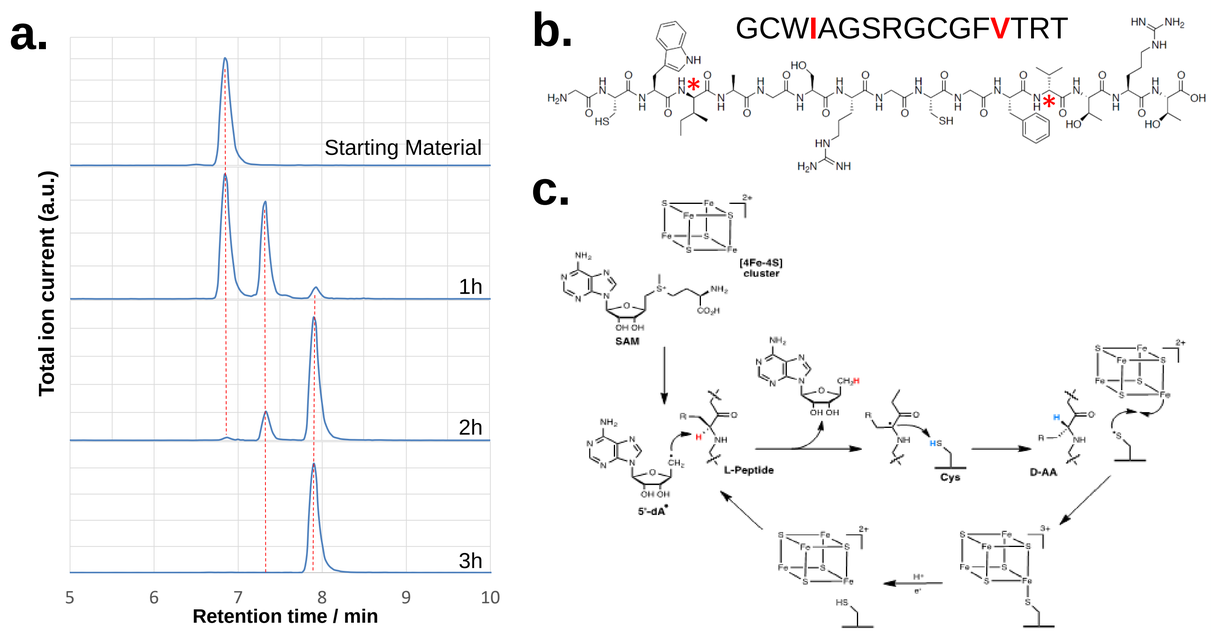

Figure 4. (**a**) LC-MS analysis of OspA core peptide and its epimerized products, (**b**) sequence and structure of core peptide and the epimerization points (red stars) and (**c**) schematic representation of proposed OspD epimerization mechanism.

4. Conclusion & Outlook

We successfully demonstrated that the reconstituted OspD epimerase is capable of converting the OspA substrate to the epimerized product *in vitro*. Our results imply that rSAM epimerases can be used as molecular tools to introduce structural diversity into their natural peptides, therefore providing an innovative platform for peptide drug discovery. Physical and biochemical characterization of the epimerized peptides will follow to identify the epimerized amino acids and to test protease resistance and antimicrobial activity.

We thank Gion-Flurin Michael and Ivana Kroslakova for technical support with LC-MS and MALDI-TOF-MS. RB thanks for financial support from ZHAW.

References: [1] *Drug Discov. Today* **2015**, *20*, 122; [2] *Annu. Rev. Microbiol.* **2004**, *58*, 458; [3] *Science* **2012**, *338*, 378; [4] *Angew. Chem. Int. Ed.* **2014**, *53*, 8503; [5] *Nat. Chem.* **2017**, *9*, 698; [6] *J. Biol. Chem.* **2001**, *277*, 1680; [7] *Angew. Chem. Int. Ed.* **2017**, *56*, 762