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COMMUNICATION

Development of a Simple High-Throughput Assay for Directed Evolution of Enantioselective Sulfoxide Reductases

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We report on the development of high-throughput fluorogenic assay that can streamline directed evolution of enantioselective sulfoxide reductases. As a model, we have evolved methionine sulfoxide reductase A (MsrA) to expand its limited substrate scope. The resulting mutant MsrA can resolve a range of new challenging racemic sulfoxides with high efficiency including the pharmaceutically relevant albendazole sulfoxide. The simplicity and the level of throughput is suitable for the future screening of metagenomic libraries for the discovery of new enzymes with similar reactivities.

The application of enzymes as biocatalysts for preparation of high value chemicals and pharmaceuticals has experience a rapid development in the past decades.[1–3] The progress of the field was enabled by inception of techniques of recombinant DNA and DNA amplification.[4,5] These technologies allowed for the first time to obtain a large variety of enzymes from different sources in sufficient quantities and purity. The next breakthrough in the field of biocatalysis was the development of the methods of directed evolution that were pioneered by Arnold and others in the early 1990s.[6–13] Utilization of DNA libraries of given enzymes enables a researcher to create millions of variants of a catalyst within a few days, which is in its efficiency unsurpassed by development of chemical catalysts. In the following screening process, the functional promiscuity of the enzyme variants enables the new reactivities to be selected. However, in spite of the success of the method, the screening process still remains a significant bottle neck of this whole enzyme evolution approach.

We have previously developed an efficient method for deracemization of chiral sulfoxides,[14] which utilizes natural enzyme methionine sulfoxide reductase A (MsrA) for the kinetic resolution of sulfoxides.[15,16] We were thus able to deracemize a wide range of substrates. The nature of the substituent at the R^1 site of the sulfoxide moiety does not significantly affect the activity of the enzyme but the only tolerated substituents at the R^2 site are methyl and ethyl groups (Scheme 1). Sterically more demanding substituents (e.g., propyl group) cause a dramatic decrease of the reaction rate. Although we were able to obtain valuable chiral synthons in a practically enantiopure form, the substrate limitations prevent the method from utilizing more complicated and pharmaceutically relevant chiral sulfoxides, such as Armodafinil and Albendazole-(*R*)-sulfoxide).[17–20] In order to deal with this limitation we resolved to perform the directed evolution of wild type MsrA. In spite of the fact that 3D NMR structure of MsrA (*E. coli*) is known,[21] we aimed at the development of a high-throughput assay that would allow a general and unbiased screen of a large sequence space of the library of MsrA random mutants rather than focused site-directed mutagenesis. Such a method would be applicable for evolution of enzymes with similar reactivities without a priori knowing the 3D structure or the positioning of the active site. Furthermore, in the reverse mode, it could serve for a quick active site mapping of sulfoxide reductases of unknown structure.

Herein, we report on the development of high-throughput fluorogenic assay for directed evolution of sulfoxide reductases.

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† Footnotes relating to the title and/or authors should appear here.
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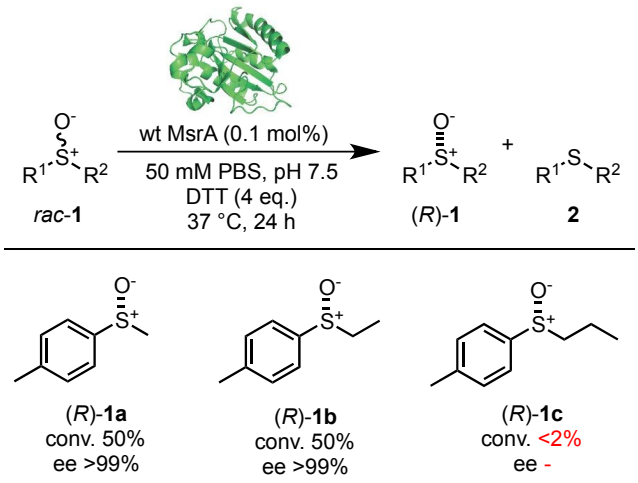
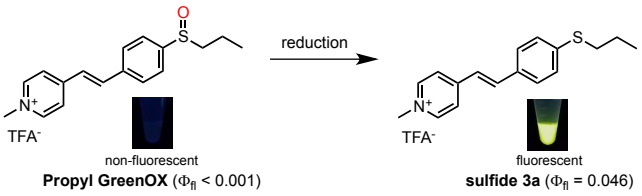


Scheme 1 Kinetic resolution of sulfoxides with wild-type MsrA and its substrate limitations.

Typically, the screening approaches with highest throughput in directed evolution of enzymatic activity involve chromogenic or fluorogenic substrates that allows rapid and efficient evaluation of the individual variants.[22] However, there is only a handful of such substrates and these allow to screen only a limited number of reactivities (most commonly hydrolysis).[23,24]Thus, we utilized our expertise in the development of fluorogenic probes for sulfoxide reductases and designed a new, highly sensitive probe we call propyl GreenOx (Scheme 2).[25,26] The probe is synthesized in two simple steps and shows negligible fluorescence (Φ_{fl} <0.001). However, upon reduction of the sulfoxide moiety to sulfide, a marked increase of the fluorescent signal is observed (Φ_{fl} = 0.046, λ_{max} = 545 nm) in both aqueous and non-aqueous environment. It has to be noted that during the preparation/submission process of this article another lab has reported similar fluorescent probes.[27]



Scheme 2 A fluorescent probe design for sensing the activity of sulfoxide reductases.

Given the substrate scope of natural MsrA, we hypothesized that propyl GreenOx would be a good substrate for the directed evolution of the enzyme. Indeed, when tested with the wild type MsrA (0.5 mol%), the reactivity at four hours was negligible (see SI). Also, methyl GreenOx variant of the probe was prepared and tested with wt MsrA. As expected, a strong fluorescent signal was observed, since the wt MsrA efficiently reduces the methyl sulfoxide moiety. In addition, the probe was found to be well permeable to *E. coli* cells, which makes it favourable for a simple assay development. Therefore, we tested methyl GreenOx on the *E. coli* colonies overexpressing wt MsrA on agar plates. The buffer solution of the probe was simply sprayed on the plates with colonies; a strong glow of those colonies that overexpress wt MsrA was observed. By contrast, *E. coli* colonies without the plasmid for wt MsrA overexpression showed no marked increase of fluorescence with methyl GreenOx, which proved the feasibility of the assay. In the next step, the wt MsrA gene was diversified by error-prone PCR and the resulting library was cloned and transformed into electrocompetent *E. coli* cells. The presence of mutations in the gene (on average 3 random mutations per gene) was confirmed by sequencing. For the proof-of-principle experiment, five plates with roughly 20 000 colonies with mutant MsrAs were obtained. Then, the plates were homogenously sprayed with the solution of propyl GreenOx. After 30 min incubation at room temperature, five colonies developed a significant yellow fluorescence (Figure 1). These were harvested, re-plated and re-assayed. Two of the five clones developed a strong yellow fluorescence after only 10 minutes. These clones termed clone 1 and clone 2 were further analysed.

Sequencing of the clones showed that clone 1 contains mutations R18C, F52L and Q109H and clone 2 mutations F52L, S106N and Q157V. Strikingly, both clones share the same amino acid mutation of phenylalanine 52 to leucine that was however caused by a different mutation in the same codon (clone 1: TTC to CTC, clone 2: TTC to TTA). We expressed both clones and measured the kinetics of reduction with propyl GreenOx. As compared to the wild type MsrA, both clones showed a dramatic increase in the reactivity (see ESI). Virtually the same kinetics of both clones led us to a hypothesis that the mutation F52L might be the major determinant of the increased reactivity. Phenylalanine 52 is located directly in the active site of the enzyme and creates a hydrophobic wall. Thus, its mutation to smaller but still hydrophobic leucine might provide more space for accommodation of bulkier substituents on the sulfoxide moiety (R^2), while not interfering with protein folding. In order

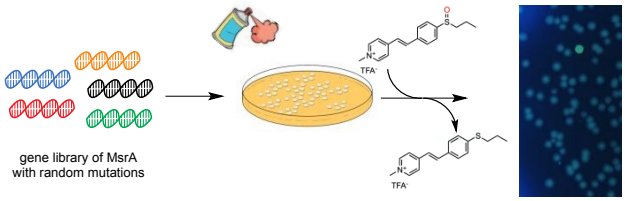


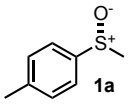
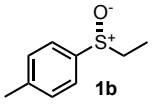
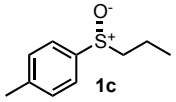
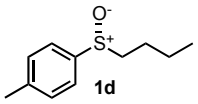
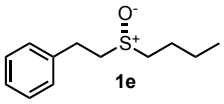
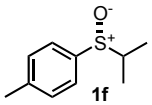
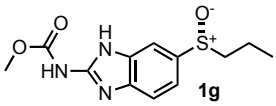
Fig. 1 A schematic representation of a high-throughput assay for the directed evolution of MsrA. The left part shows a snapshot of a real plate from the selection.

to test this hypothesis, we created a clone with a single mutation F52L by site-directed mutagenesis. This clone showed basically the same kinetics of reduction of propyl GreenOx as the clone 1 and 2, which confirmed the hypothesis that the F52L mutation is solely responsible for the change in the activity of the enzyme.

Next, we tested the wt MsrA and the clone F52L in reactions with substrates **1a**-**1g** (Table 1). While wt MsrA expectedly resolved only substrates **1a** and **1b** with methyl and ethyl substitution, the clone F52L was able to successfully resolve also

substrates **1c** and **1d** with significantly larger propyl and butyl substituents in the sulfoxide moiety. Also, the challenging substrate **1e** with two bulky alkyl groups on the sulfoxide was successfully resolved with an excellent enantioselectivity by using the F52L clone. Delightfully, also the pharmaceutically relevant albendazole-(*R*)-sulfoxide (**1g**) that is the active metabolite of the essential anti-parasitic agent albendazole, can now be obtained with high enantioselectivity (94% ee). Branching at the alpha carbon of the sulfoxide moiety (**1f**) seems to set the limits to the significantly expanded substrate scope of the F52L clone.

Table 1 Substrate scope of kinetic resolution with wt MsrA and F52L MsrA mutant.[a]

Entry	Product	wt MsrA conversion (ee)	F52L MsrA conversion (ee)
1	 **1a**	50% (99%)	50% (99%)
2	 **1b**	50% (99%)	50% (99%)
3	 **1c**	<3%	50% (99%)
4	 **1d**	<3%	50% (99%)
5	 **1e**	<3%	50% (99%)
6	 **1f**	<3%	<3%
7[b]	 **1g**	6% (6%)	49% (94%)

[a] Reaction conditions: substrate (3.2 μmol), MsrA (0.5 mol%), DTT (12.8 μmol), PBS (0.5 mL), 24 h. [b] 1.2 mol% of MsrA used.

It should be noted that the evolution assay was performed with a racemic probe GreenOx. We hypothesized that the structural requirements in the active site of MsrA would retain the enantioselectivity even in the more reactive mutants, which indeed was the case. Nevertheless, the new assay can be easily adjusted to implement a screening for enantioselectivity as well. As we have previously demonstrated, utilization of an enantiomerically pure fluorogenic probe with the non-fluorogenic competitor of the opposite chirality can easily address this issue.[28] As some sulfoxide reductases can act also in the reversed fashion as oxidases,[29,30] this simple assay would also be applicable for evolution of enzymes for enantioselective oxidation of prochiral sulfides. In general, a similar approach can be applied for evolution of any enantioselective enzymes that can catalyse both forward and reverse reactions (e.g., ketoreductases and transaminases, etc.).

In conclusion, we have developed a new simple and widely applicable high-throughput screening assay for the directed evolution of enantioselective sulfoxide reductases. In the proof-of-principle experiment we have evolved natural MsrA. The resulting mutant MsrA has a significantly broader substrate scope, enabling to resolve a range of challenging chiral sulfoxide with high efficiency and enantioselectivity, including the pharmaceutically relevant albendazole-(*R*)-sulfoxide (**1g**). The simplicity and high-throughput of the assay enables efficient screening of large libraries of enzyme mutants with this enantioselective reductase activity. Moreover, the level of throughput makes this method also suitable for screening of large metagenomic libraries for new enantioselective sulfoxide reductases.

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Conflicts of interest

There are no conflicts of interest.

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