Halogenase Activity

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A High-Throughput Fluorescence Assay to Determine the Activity of Tryptophan Halogenases

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In memory of Klaus Burger

Abstract: *Biocatalytic halogenation with tryptophan halogenases is hampered by severe limitations such as low activity and stability. These drawbacks can be overcome by directed evolution, but for screening large mutant libraries, a facile high-throughput method is required. Therefore, we developed a quantitative halogenase assay based on a Suzuki–Miyaura cross-coupling towards the formation of a fluorescent aryltryptophan. The technique was optimized for application in crude E. coli lysate without intermediary purification steps, and was used for quantitatively monitoring the formation of halogenated tryptophans with high specificity by facile fluorescence screening in microtiter plates. This novel screening approach was exploited to engineer a thermostable tryptophan 6-halogenase. Libraries were constructed by error-prone PCR and selected for improved thermal resistance simply by fluorogenic cross-coupling. Our method led to an enzyme variant with substantially increased thermal stability and 2.5-fold improved activity.*

Nature has evolved a comprehensive toolkit for biocatalytic C–H functionalization that can also be harnessed for chemoenzymatic synthesis under benign reaction conditions.[1] Among them, biocatalytic halogenation has emerged as a topic of major interest as the enzymatic pathway enables sustainable syntheses of aryl halides that only require oxygen, the cofactor $FADH_2$, and halide salts for site-selective halogenation.[2] Groundbreaking research by the groups of van Pée and Walsh focused on the discovery and elucidation of flavin-dependent tryptophan halogenases, revealing an intriguing halogenation mechanism.[3–7] Their findings paved the way for exploiting these biocatalysts in the large-scale halogenation of L-tryptophan (Trp) and related derivatives.[8,9] However, their application is hampered by low efficiency and insufficient stability. In 2015, we were able to improve the stability of the Trp 7-halogenase RebH by formation of cross-linked enzyme aggregates that enable scalable regioselective bromination, currently up to gram scale.[10] Likewise, Lewis et al. used protein engineering to improve the thermostability and lifetime of RebH, which led to a more resistant mutant endowed with improved thermal resistance, but decreased turnover.[11] Recent approaches contributed to enlarging the substrate scope towards different aryl substrates by rational and random engineering.[12,13] However, directed halogenase evolution initially suffered from rather low throughput because library screening relied on UHPLC separation. Usually, efficient and robust screening approaches based on colorimetry or fluorescence readout are a mandatory prerequisite to successfully evaluate larger mutant libraries in microtiter plates, with cell sorters, or directly on agar plates.[14–18] As an alternative to HPLC-based halogenase assays, quinone–amine coupling can be used to colorimetrically monitor arylamine halogenation.[19] Recently, Lewis et al. introduced a MALDI-ToF MS assay with deuterium-labeled tryptamine to alter the regioselectivity of RebH by means of directed evolution.[20]

Owing to the limited scope of these screenings, we felt encouraged to develop a more universal assay applicable in cell lysate by specific modification of the introduced halogen substituent, independent from the presence of other functional groups. Palladium-catalyzed cross-couplings thus came into focus as such reactions have been proven to be sufficiently bioorthogonal,[21,22] and we assumed that the resulting biaryl compounds possess distinct spectroscopic properties as previously shown by Goss et al.[23] Suzuki–Miyaura couplings (SMCs) were expected to be suitable for our purposes as the catalyst tolerates aqueous media and the employed boronic acids are stable without requiring protecting groups.[24] The groups of Goss and O'Connor have successfully utilized SMCs for natural product derivatization to obtain functionalized peptides or indole alkaloids.[25,26] Very recently, we published a one-pot synthesis of aryl-substituted tryptophans in water by in situ bromination, SMC, and N^{α}-Boc protection.[27] At the same time, Lewis et al. employed crude extracts of enzymatic halogenation for arylation and Buchwald–Hartwig amination or alkoxylation.[28] In a more comprehensive study, Micklefield et al. addressed catalyst-directed C–H functionalization by biohalogenation and SMC in a one-pot process. By making use of compartmentalization, they developed a promising method to overcome the inherent incompatibility between halogenase and metal catalyst.[29]

We began the assay development with the synthesis of different aryltryptophans to identify suitable screening probes (Figure 1). The C7-substituted compounds **6–9** were formed in the presence of Na_2PdCl_4, the Buchwald ligand SPhos,[30,31]

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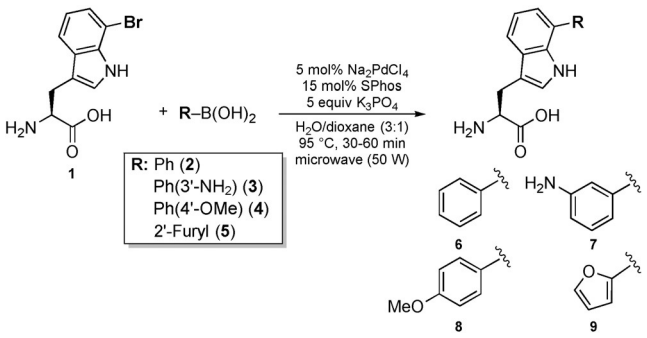

Figure 1. Synthesis of C7-aryl-substituted L-tryptophan derivatives.

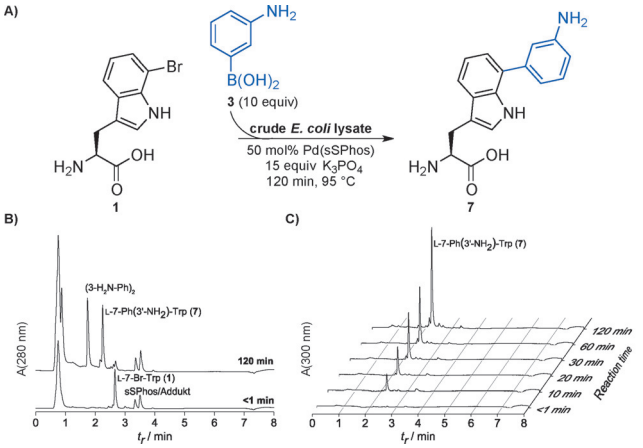

Figure 2. A) SMC of halide **1** and boronic acid **3** in *E. coli* lysate using Pd(sSPhos)[31] as the catalyst. B) The RP-HPLC diagram of the cross-coupling in crude bacterial lysate at $\lambda = 280$ nm shows approximately full conversion of **1** ($t_R = 2.7$ min) after heating for 120 min at 95 °C. The successful synthesis of **7** ($t_R = 2.2$ min) was confirmed by LC-MS and comparison with an authentic standard. Traces of chlorotryptophan remained in the lysate, and homocoupling product 3,3′-diaminodiphenyl was identified as the major side product. C) The time course of the coupling analyzed at $\lambda = 300$ nm shows that solely the coupling product **7** ($t_R = 2.2$ min) was detected in this spectral range, and its formation can thus be monitored quantitatively. sSPhos = sodium 2′-dicyclohexylphosphino-2,6-dimethoxy-1,1′-biphenyl-3-sulfonate hydrate.

2 equiv boronic acid, and K_2CO_3 or K_3PO_4. UV spectroscopic analysis revealed a strong absorption below $\lambda = 225$ nm for Trp and most of its derivatives (Table 1; see also the Supporting Information, Figure S1). Moreover, Trp and **1** gave rise to a maximum at $\lambda = 280$ nm whereas the maxima of **6**–**9** were shifted to $\lambda = 300$–320 nm, indicating the strong influence of the additional aryl moiety (Table 1). Although **9** exhibited the largest red shift, aniline derivative **7** was examined in detail as a promising target for the screening assay owing to its enhanced water solubility. Moreover, the NH_2 group enables the detection of otherwise hardly ionizable indoles to identify novel halogenated compounds by LC-MS.

Table 1: Summary of the specific UV absorbance maxima of L-tryptophan and C7-aryl-substituted derivatives above $\lambda = 250$ nm at pH 7 and 25 °C.

R–B(OH)$_2$	Compound	λ_{max} [nm]
–	L-tryptophan	280
–	L-7-bromotryptophan (**1**)	284
2	L-7-phenyltryptophan (**6**)	300
3	L-7-(3′-aminophenyl)tryptophan (**7**)	300
4	L-7-(4′-methoxyphenyl)tryptophan (**8**)	297
5	L-7-(2′-furyl)tryptophan (**9**)	317

However, SMC in crude bacterial lysate, proved challenging. We started with *E. coli* lysate containing RebH for bromination of 5 mM Trp, which gave product **1** with > 99 % conversion (Figure S2). The crude mixture containing **1** was utilized as a model system for the SMC (Figure 2 A). Stepwise optimization (see the Supporting Information for details) finally resulted in a robust procedure that could be applied as the screening reaction in lysate. Improved reaction conditions gave > 98 % conversion into **7** after 120 min as indicated by HPLC and LC-MS while only minor amounts of chlorotryptophan remained in the reaction mixture with oxygen-induced homocoupling as the dominant side reaction. The HPLC peak at 3.3 min corresponds to water-soluble SPhos whereas an adduct of the ligand and **3** was observed as a side product at 3.5 min (Figure 2 B; see also Figure S6). We additionally monitored the reaction progress by measuring the UV absorbance at $\lambda = 300$ nm (Figure 2 C). At this wavelength, the specific formation of chromophore **7** can be monitored while starting material and other contaminants are not detectable in this region, providing a valuable starting point for further assay development.

Additional investigations of the fluorescence characteristics of **7** revealed an emission maximum at $\lambda = 430$ nm upon excitation between $\lambda = 280$ and 300 nm whereas tryptophan was non-fluorescent in this spectral range (Figure 3 A; see also Figure S7). It is noteworthy that the fluorescence of bromotryptophan (**1**) was substantially weaker than that of other Trp derivatives. Therefore, a fluorescence assay simply based on the introduction of the Br substituent was inappropriate owing to the high background signal of cellular contaminants.

Regarding the high-throughput screening (HTS), the concentration dependence of the assay was investigated in a microtiter plate format. Lysate samples containing either Trp or **1** were incubated with boronic acid **3** under the previously optimized SMC assay conditions and finally analyzed by fluorescence spectroscopy using a microplate reader (Figure 2 A). The fluorescence signal increased with the concentration of brominated amino acid **1** whereas samples containing Trp were only weakly fluorescent (Figure 3 B). As a further proof of concept, we tested RebH lysates containing both Trp and **1** in varying molar ratios. The emission at $\lambda = 430$ nm was not influenced by the presence of non-brominated Trp, leading to a linear correlation between fluorescence and product concentration. To broaden the applicability of our approach, we applied the assay towards the Trp 5- and 6-halogenases PyrH[32] and Thal,[33] respectively. The C5- and C6-aryl-substituted isomers of **7** were synthesized as described above. Despite the less favorable

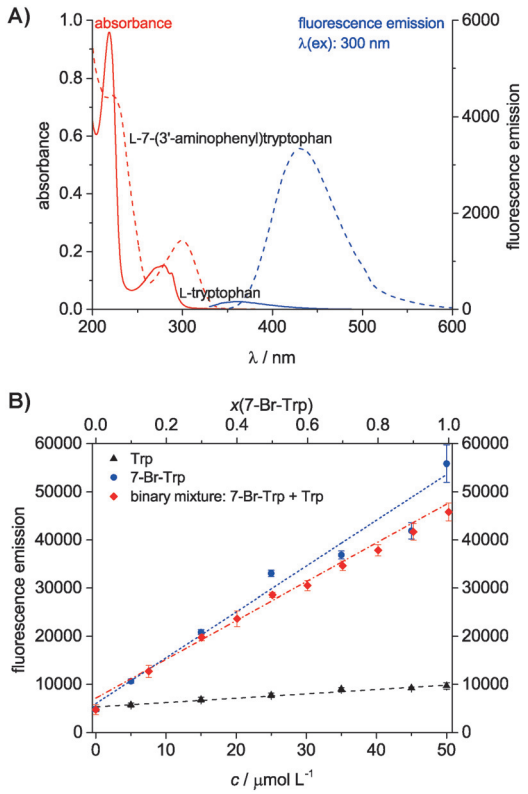

Figure 3. A) UV absorbance (red) and fluorescence emission (blue) spectra (λ_{ex} = 300 nm). The absorption spectrum of **7** (-----) is bathochromically shifted with respect to that of Trp (——) with λ_{max} = 300 nm and a red-shifted fluorescence maximum at 425 nm, whereas Trp is only weakly fluorescent in this spectral range. B) The fluorescence calibration plot (λ_{ex} = 300 nm, λ_{em} = 430 nm) confirms the specificity for the brominated amino acid in the HTS lysate assay (bottom axis: Trp or bromotryptophan concentration), even with a binary mixture consisting of Trp and its brominated derivative (top axis: molar ratio of 7-bromotryptophan).

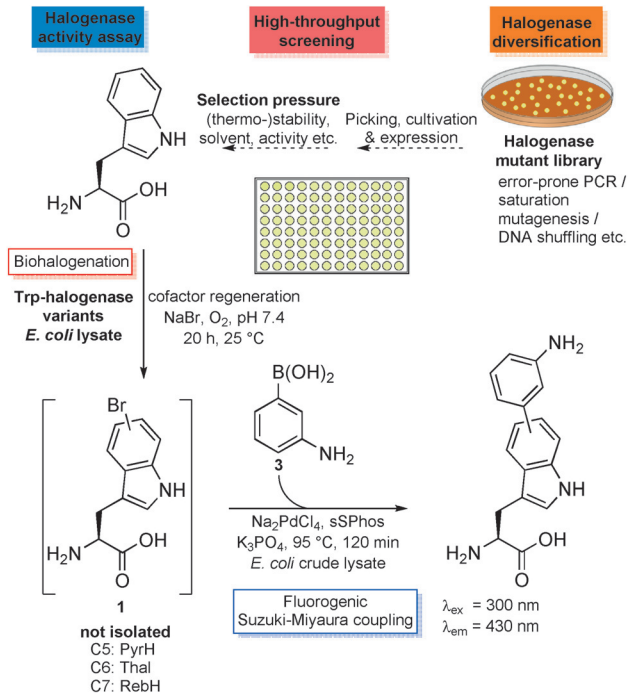

Figure 4. Halogenase engineering assay based on SMC to determine the halogenase activity. The procedure simplifies the selection process of mutant libraries.

coupling of **3** in the electron-rich C5 position and weaker fluorescence emission, the C5 and C6 analytes were also found to be appropriate to monitor PyrH and Thal activity in lysate (Figures S8–S10).

Finally, we strived to engineer a thermostable tryptophan 6-halogenase by using fluorogenic SMC and set up a HTS strategy (Figure 4). To simplify the assay procedure, sealed microtiter plates were placed in a drying oven at 95 °C for the cross-coupling, which provided increased throughput. Along this line, a library of Thal mutants with an average of two mutations was generated by error-prone PCR, and the resulting colonies were picked and cultivated in a microtiter plate to express the gene variants. After cell lysis, the halogenase libraries were treated for 20 min at 49 °C to select for enhanced thermostability because previous experiments had shown that wild-type Thal becomes inactive above 45 °C. Subsequently, the heat-shocked Thal libraries were utilized for bromination of 5 mM Trp at 25 °C followed by HT-SMC to monitor the residual conversion of substrate in the microtiter plates. In parallel, control plates without prior heat shock treatment were screened to eliminate expression mutants. Screening of 300 transformants gave one Thal variant with conspicuously higher conversion. Sequencing of the corresponding plasmid DNA unveiled two mutations, S359G and K374R (termed Thal-GR), whose influence was studied further. As the introduction of a rare AGA base triplet encoding R374 affected the protein yield, it was substituted by the more frequent CGT codon, leading to high levels of gene expression similar to wt Thal (Figure S11).

Purified protein samples were incubated between 40.0 and 62.5 °C for 20 min and assayed for bromination at 25 °C to assess enzyme denaturation. We found that the half-maximal inactivation temperature (T_M) increased from 47 °C (wt Thal) to 57 °C (Thal-GR; Figure 5A). Whereas the activity of wt Thal dropped below 5% above 49 °C, the thermostable variant Thal-GR still reached >99% conversion at 49 °C and, finally, 71% at 56 °C (Figure 5B). To examine the halogenase stability, enzyme samples were incubated at 40 °C for different periods of time, and the biocatalysts were subsequently used for bromination at room temperature. Whereas the wt enzyme suffered from a significant loss of activity after 2–3 h, as evidenced by precipitation, Thal-GR withstood 6 h of incubation, still giving 69% conversion (Figure 5C). Examination of the enzyme activity revealed a rapid increase in the amount of brominated product within 90 min catalyzed by Thal-GR, whereas wt Thal reached only 59% substrate conversion over the observed time range (Figure 5D). It is noteworthy that the specific activity of the mutant Thal-GR was 2.5 times higher than that of wt Thal as determined from the linear range. This result clearly indicates that a more efficient halogenation catalyst has been generated. Furthermore, we confirmed by NMR analysis that the mutations did not alter the regioselectivity of Thal, giving L-6-

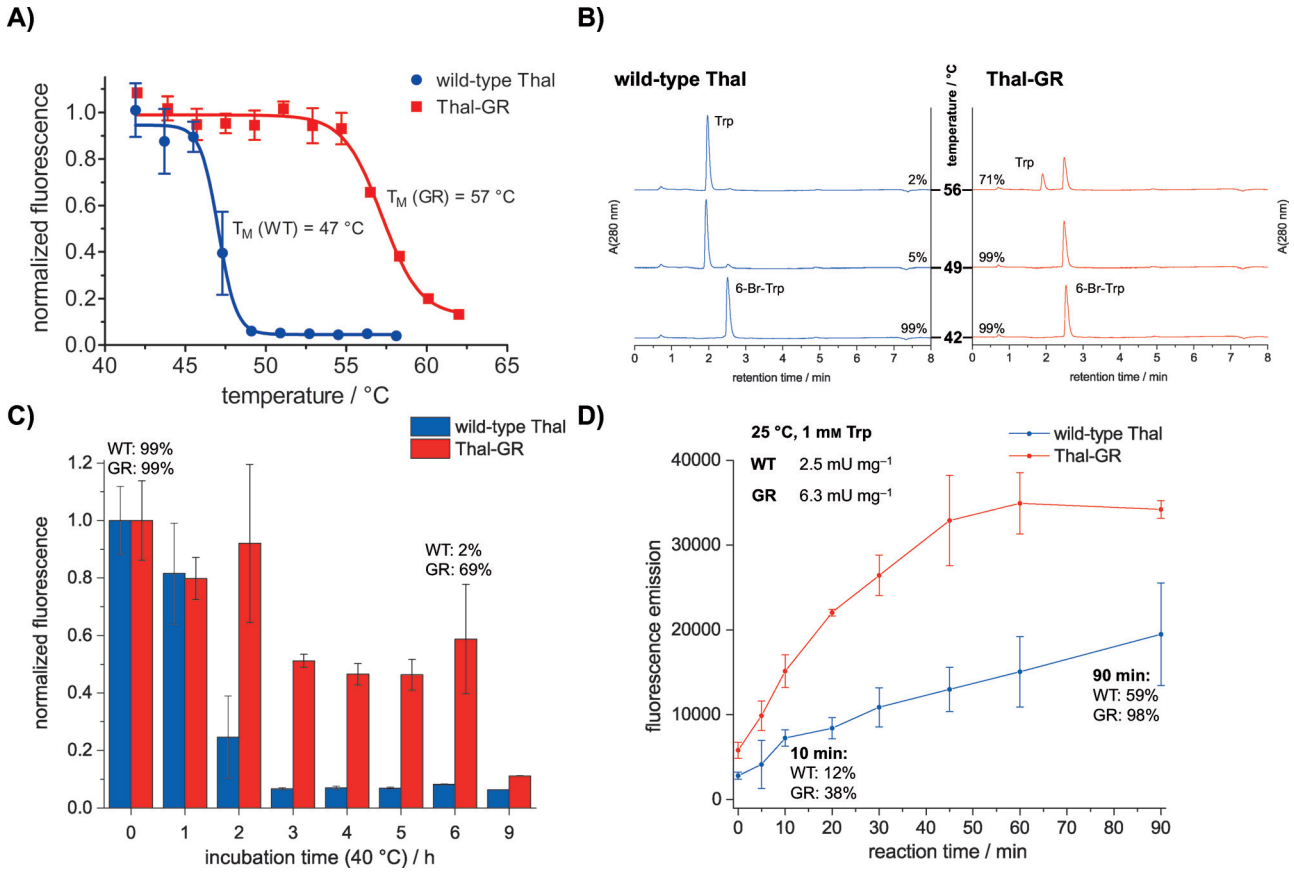

Figure 5. Determination of the catalyst properties of wt Thal and Thal-GR. A) Fluorescence data correlating with the final conversion after incubation at elevated temperatures for 20 min were normalized to a control reaction without prior heat shock that reached full conversion. Sigmoidal regression shows that the denaturation point of Thal-GR is 10 K higher than that of wt Thal. B) RP-HPLC chromatograms of halogenation reactions upon thermal incubation confirm the improved thermal resistance of Thal-GR. C) The results of long-term incubation at 40 °C showcase the prolonged lifetime of Thal-GR for at least 6 h. D) The time dependence of the reaction progress at 25 °C shows that Thal-GR is approximately 2.5 times more active, giving full conversion in 90 min. Reaction conditions: A/B/C: 5 mM Trp, 30 μM (A) or 11 μM (B/C) Thal; D: 1 mM Trp, 7 μM Thal. General conditions: 2.5 U mL^{-1} PrnF, 1 U mL^{-1} ADH, 10 μM FAD, 1 mM NAD^+ along with 30 mM NaBr and 5% *i*PrOH at 25 °C. The reaction mixtures were analyzed by SMC according to Figure 4. The relative conversion was determined by HPLC by determining the ratio of the peak areas of Trp and 6-bromotryptophan.

bromotryptophan as a single isomer (see the Supporting Information for details).

In conclusion, halogenase engineering requires a facile high-throughput assay to overcome general drawbacks and enable the application of halogenases in synthesis. Therefore, our efforts concentrated on developing a robust enzyme test based on cross-coupling with a spectroscopic readout for screening in cell lysate. We identified the coupling product of 3-aminophenylboronic acid (**3**) and bromotryptophan as an appropriate fluorogenic probe and established a halogenase activity assay with high specificity for 5-, 6-, and 7-bromotryptophan analyte in microtiter plates. In contrast to recently published halogenase assays,[19,20] the fluorogenic SMC enables facile and rapid library screening while maintaining a broad application scope. Incubation in an oven leads to nearly unlimited throughput without requiring specialized laboratory equipment. We presume that our assay is easily tunable towards several substrates after calibration with a suitable standard. With the novel HTS in hand, we found a thermostable Trp 6-halogenase variant with a T_M of 57 °C by random mutagenesis. The mutant S359G/K374R displayed significantly higher activity and prolonged catalyst lifetime. Hence, the applicability of the novel cross-coupling assay has been demonstrated, and our results will contribute to tackle general deficiencies of enzymatic halogenation. We will continue to focus on enzyme engineering to identify robust halogenases that are capable of large-scale C–H functionalization and can be integrated in multistep syntheses.

Keywords: cross-coupling · directed evolution · halogenases · high-throughput screening · one-pot processes

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