

Enzymatic Halogenation

Enzymatic Halogenation of Tryptophan on a Gram Scale**

Marcel Frese and Norbert Sewald*

Abstract: Halogenated arenes are important building blocks in medicinal and agrochemistry. Chemical electrophilic aromatic halogenation requires molecular halogen, whereas FAD-dependent halogenases form halogenated arenes with high regioselectivity while only halide salts and O_2 are required. This reaction proceeds at room temperature in aqueous media. However, enzymatic halogenation is considered inefficient, mainly because halogenases are not stable. Thus, the preparative application remained elusive. We were able to show that the long-term stability and, hence, the preparative efficiency of the tryptophan-7-halogenase RebH can be significantly improved by immobilization together with the other enzymes required for cofactor regeneration. We established a facile scalable method suitable for the halogenation of tryptophan and its derivatives on a gram scale using a solid, multifunctional, and recyclable biocatalyst; this immobilization strategy might also be applicable for other FAD-dependent halogenases.

Halogenation is a common reaction in organic synthesis, yet the regioselective introduction of halogen substituents at mechanistically less favored positions remains a challenging task. Aryl halides are important intermediates in the chemical, agrochemical, and pharmaceutical industries because they can be modified readily by nucleophilic substitution and metal-catalyzed cross-coupling reactions.^[1] The chemical incorporation of halogen substituents is an environmentally hazardous process that requires elemental chlorine or bromine, often in combination with Lewis acids and may also lead to the formation of byproducts because of ambiguous regioselectivity. In nature, enzymatic strategies have evolved for the halogenation of organic metabolites under much milder conditions, making use of benign halide salts and oxygen at 25 °C and pH 7. FAD-dependent halogenases form the major class of enzymes responsible for regioselective, carrier-free halogenation in nature. Representatives of this class are the Trp-7 halogenase PrnA^[2] and its close relative RebH from *Lechevalieria aerocolonigenes*.^[3] L-Tryptophan is chlorinated regioselectively at the electronically unfavored C7 position of the indole ring. A flavin reductase supplies FADH₂, which is oxidized in the active site of the halogenase

by molecular oxygen. The resulting flavin hydroperoxide undergoes nucleophilic attack by a halide ion to form hypohalous acid, which passes through a 10 Å long channel from the FAD binding site to the tryptophan binding site.^[4–7] Due to the sandwichlike binding of L-tryptophan inside the active site of the halogenase, only the less activated C7 position becomes available for chlorination or bromination.^[7,8] The conserved lysine residue K79 is oxidized selectively by hypohalous acid and the halogenation is brought about by the actual halogenating species, a long-lived N-haloamine intermediate.^[9] Beside the Trp-7 halogenase RebH, C5^[10] and C6^[11] Trp-halogenases have also been described in the literature.

Enzyme-catalyzed reactions are well-known for their high stereo- and regioselectivity.^[12,13] Consequently, biocatalysis has become an emerging field in biotechnology, especially in terms of green and sustainable chemistry. Moreover, owing to the high selectivity of enzymatic reactions, the application of biocatalysts supersedes the necessity of protecting or activating groups, leading to more efficient synthetic routes. However, enzymes like halogenases suffer from low stability and low activity, especially under non-native reaction conditions in the presence of high substrate concentrations.

Enzyme immobilization has proven to be an amenable solution to circumvent stability problems,^[14] and it also facilitates recycling and easy removal of the biocatalyst. Although different immobilization strategies have been developed in the past, cross-linked enzyme aggregates (CLEAs) have emerged as carrier-free catalysts with outstanding benefits owing to their simplicity. Briefly, the enzyme of choice is precipitated upon addition of ammonium sulfate or polyethylene glycol and then lysine residues on the enzyme surface are cross-linked by bifunctional molecules like glutaraldehyde. The crucial advantage of this methodology is the possibility of combining purification and immobilization in one step, which also makes this approach applicable for crude protein extracts.

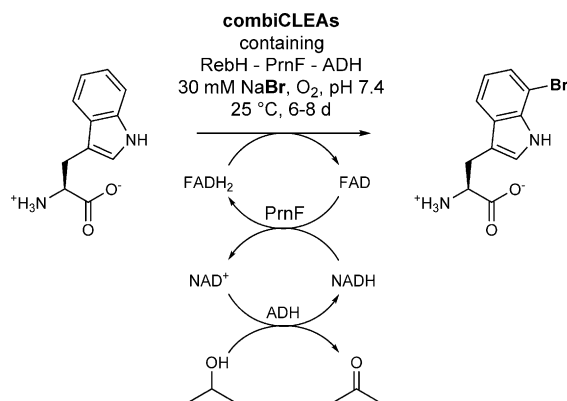
With respect to halogenases, several studies determined low kinetic constants with turnover rates k_{cat} of approximately 1.0 min^{−1} and total turnover numbers (TTNs) of less than 200, which makes the practical synthetic applicability of halogenases elusive.^[2,3,6,9,11,15–17] Up to this point, halogenases have mainly been studied on an analytical scale to determine the substrate scope,^[18] whereas preparative enzymatic halogenation still requires optimization. Recently, Payne et al. used *E. coli* lysate containing overexpressed RebH from more than 10 L of cultivation medium to obtain less than 90 mg of chlorinated tryptophan in moderate yields.^[15] In addition, large amounts of contaminants in *E. coli* lysate containing overexpressed halogenases hinder purification of the halogenation product. In particular residual chloride ions from the cultivation media interfere with bromination reactions.

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We embarked on a project focusing on the development of a facile enzymatic halogenation system that can be applied on a multigram scale without requiring large expression volumes. Hence, the idea was to concomitantly immobilize L-tryptophan-7-halogenase RebH and the necessary auxiliary enzymes by precipitation and cross-linking in order to obtain one solid biocatalyst as multifunctional cross-linked enzyme aggregates, so-called combiCLEAs (Scheme 1). The



Scheme 1. Cross-linking of precipitated Trp-7-halogenase RebH, flavin reductase PrnF, and an alcohol dehydrogenase from *Rhodococcus* sp. leads to multifunctional, recyclable cross-linked enzyme aggregates (CLEAs), which can be applied for the regioselective halogenation of L-tryptophan also on the gram scale.

catalyst should be removable by filtration for the recyclable usage of RebH. *E. coli* lysate from 1.5 L of culture containing the overexpressed halogenase RebH was used without any further purification together with the required auxiliary enzymes for continuous cofactor regeneration: a flavin reductase from *Pseudomonas fluorescens* (PrnF) and an alcohol dehydrogenase from *Rhodococcus* sp. (ADH) were added. After precipitation of all three enzymes by addition of ammonium sulfate, glutaraldehyde was added at different concentrations to determine the ideal reagent ratio for cross-linking. CombiCLEAs produced at low glutaraldehyde concentrations showed the highest residual activity (Figure 1 A). Since the proposed halogenation mechanism of RebH involves a *N*-haloamine at the K79 residue that is essential for halogenation activity,^[9] the modification of this distinct residue by glutaraldehyde might be responsible for the lowered residual activity. However, it is known that excessive protein modification by glutaraldehyde in general leads to lower activities.^[19] Different cross-linking times did not lead to a significant decrease in the remaining activity. Under optimized conditions, an immobilization yield of all three simultaneously immobilized enzymes of 99% and an activity recovery of 30% was achieved. Long-term storage experiments proved RebH combiCLEAs to be stable for more than 4 months at 4 °C, whereas free, purified RebH showed a considerable loss of activity after 12 weeks (Figure 1 B).^[20]

RebH combiCLEAs can be recycled at least 10 times for the halogenation of 3 mM L-tryptophan in a batchwise manner with an average conversion of 81%, leading to more than

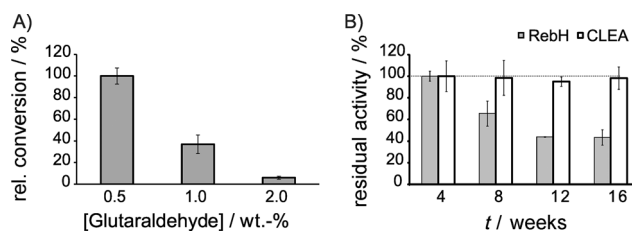


Figure 1. A) Influence of different glutaraldehyde concentrations on the cross-linking of the precipitated enzymes RebH, PrnF, and ADH. The highest conversion of L-Trp was achieved by using relatively low cross-linker concentrations of 0.5%. Since glutaraldehyde forms Schiff bases with primary amines, higher concentrations might lead to the modification of the K79 residue that is essential for halogenation activity.^[9] B) Long-term storage experiments show that RebH combiCLEAs are stable for more than 4 months, whereas free purified RebH showed a considerable loss of activity after 12 weeks.

200 mg of regioselectively brominated L-tryptophan as the TFA salt (Figure 2).

Notably, each halogenation reaction stops at a distinct time, suggesting a limitation, for example, of the cofactor regeneration by ADH. However, higher 2-propanol concentrations or in situ product removal (ISPR) of the produced acetone^[21] did not extend the catalytic activity. Also higher aeration and additional oxygen supply to the vessel did not improve the reaction.^[22] Decreasing the L-tryptophan concentration from 3 to 1 mM and increasing the volume from 30 mL to 750 mL drove the halogenation of 153 mg L-tryptophan to completion within 6 days without the need to replace the reaction buffer (Figure 3). Since the halogenation is highly regioselective, a single desalting step using reversed-phase C18 silica is sufficient for workup, leading to 248 mg of

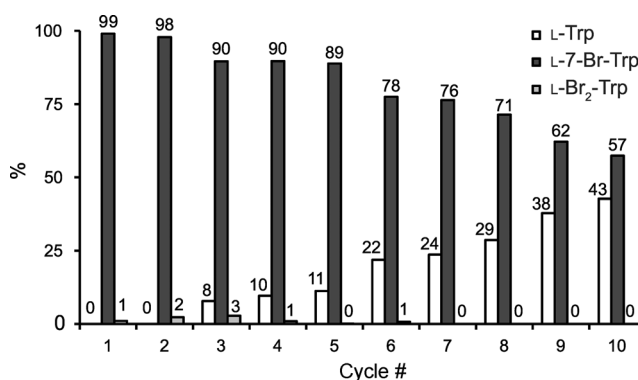


Figure 2. RebH combiCLEAs from 1.5 L of *E. coli* culture were used in batch reactions in a final volume of 30 mL containing 3 mM L-tryptophan to test their reusability. After each batch reaction, the solid biocatalyst was removed by centrifugation, washed once with buffer, and reused for the next batch in new reaction solution. RebH combiCLEAs can be recycled at least 10 times with an average conversion of 81%, leading to 204 mg of L-7-bromotryptophan (TFA salt, 0.52 mmol, 58%). After quantitative halogenation of L-tryptophan, RebH starts to form the dibrominated species as well.^[15] However, since the product of the first bromination is deactivated for further electrophilic aromatic substitution, the dibromination is very slow and occurs to only a very low extent. This issue can be easily avoided by reaction monitoring and removal of the immobilized biocatalyst.

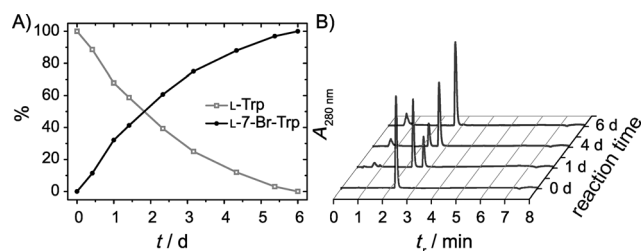


Figure 3. A) RebH was shown to brominate 154 mg of L-tryptophan within 6 days without the need to regenerate the reaction buffer when higher volumes and lower concentrations were used. A simple desalting step using RP-silica led to 248 mg of L-7-bromotryptophan-TFA. B) According to RP-HPLC analysis, L-tryptophan ($t_r = 128$ s) is converted with high selectivity to L-7-bromotryptophan ($t_r = 156$ s) without the formation of any side products.

L-7-bromotryptophan (TFA salt, 0.63 mmol, 84 %). Therefore, we were confident that the halogenation reaction could also be scaled up to the gram scale. CLEAs from 6 L of *E. coli* culture overexpressing RebH were used for the halogenation of 1 g of L-tryptophan in a final volume of 5 L. Within 8 days, the reaction proceeded to full conversion, leading to 1.813 g of L-7-Br-Trp-TFA (4.58 mmol, 92 %) in high purity after simple desalting using a plug of C18 silica.

Since we previously demonstrated the potential of RebH for the regioselective halogenation of other L-tryptophan derivatives at the C7 position, even in the presence of inactivating *ortho/para*-directing groups like 5-fluoro,^[23] we also applied the RebH combiCLEAs for the halogenation of L-5-hydroxytryptophan, as well as D-tryptophan (Table 1). Even for such substrates, the halogenation works on a preparative scale, albeit slightly lower yields were obtained.

Table 1: Halogenation of tryptophan and 5-hydroxytryptophan by RebH.^[a]

Substrate	Concentration	Product	Conv. [%] ^[b]
L-Trp	1 mM	L-7-bromo-Trp	100
D-Trp	0.25 mM	D-7-bromo-Trp	57
5-HO-Trp	0.25 mM	7-chloro-5-HO-Trp ^[c]	53

[a] Reaction conditions: 100 μ M NAD⁺, 1 μ M FAD, 15 mM Na₂HPO₄, 5 vol% 2-propanol, and 30 mM NaBr or NaCl in a final volume of 750 mL Millipore Water, pH 7.4 adjusted with H₃PO₄, 25 °C, 6 d. [b] Determined by RP-HPLC. [c] 6-Chloro-5-HO-Trp is also formed (product ratio 1:3, verified by ¹H NMR spectroscopy).

The practical applicability of FAD-dependent halogenases for preparative regioselective halogenation has been questioned because of low kinetic constants in combination with poor total turnover numbers, which limited RebH to analytical application or to low yields. Prohibitively extensive efforts were previously necessary to halogenate 100 mg of L-tryptophan, for example, by using 12 L of *E. coli* culture containing overexpressed RebH. By using RebH-PrnF-ADH combiCLEAs, we have established a facile enzymatic regioselective halogenation process that is easily scalable up to gram scale. For the first time, an active FAD-dependent halogenase was co-immobilized with all auxiliary enzymes necessary for halogenation. Owing to the dimeric structure of

RebH,^[9] the formation of CLEAs might stabilize its quaternary structure, which is also known for several other multimeric enzymes.^[24] Since numerous other tryptophan halogenases like PyrH (Trp-5-halogenase) and PrnA (Trp-7-halogenase) also form dimers in solution,^[7,8] the CLEA methodology is likely to be applicable also for these enzymes to improve their biocatalytic application. In addition, the further improvement of the mechanical properties of the combi-CLEAs by cross-linking the enzymes in presence of different additives like siloxanes^[25,26] and magnetic functionalized beads^[27] might enhance the catalytic efficiency even more.

Although enzymatic halogenation is still in its infancy because of its limited substrate scope, the preparative utilization of immobilized RebH for the regioselective bromination of L-tryptophan on the gram scale laid the foundation for efficient biocatalytic halogenation. As a consequence, our work now focusses on the expansion of the substrate scope from tryptophan to other (hetero-)aromatic rings by means of directed evolution.

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