Biocatalysis

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Enzymatic Conversion of Flavonoids using Bacterial Chalcone Isomerase and Enoate Reductase**

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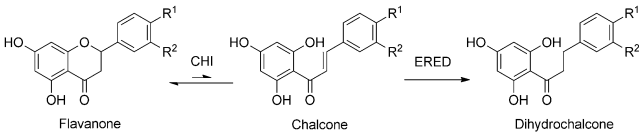
Abstract: *Flavonoids are a large group of plant secondary metabolites with a variety of biological properties and are therefore of interest to many scientists, as they can lead to industrially interesting intermediates. The anaerobic gut bacterium Eubacterium ramulus can catabolize flavonoids, but until now, the pathway has not been experimentally confirmed. In the present work, a chalcone isomerase (CHI) and an enoate reductase (ERED) could be identified through whole genome sequencing and gene motif search. These two enzymes were successfully cloned and expressed in Escherichia coli in their active form, even under aerobic conditions. The catabolic pathway of E. ramulus was confirmed by biotransformations of flavanones into dihydrochalcones. The engineered E. coli strain that expresses both enzymes was used for the conversion of several flavanones, underlining the applicability of this biocatalytic cascade reaction.*

Flavonoids are polyphenolic compounds that occur in plants; they are involved in plant coloration and act as biochemical sensing molecules, but also have important roles as antioxidants[1] and flavor enhancers.[2] Their biosynthesis in plants is well documented in the literature, and the basic scaffold is formed from malonyl and coumaroyl precursors in the presence of 4-coumaryl-CoA ligase and a chalcone synthase. In the next step, chalcone isomerase (CHI) catalyzes the stereospecific formation of the tricyclic flavanones such as naringenin (Scheme 1). These are further functionalized by other enzymes, such as hydroxylases or glycosidases, to create the diverse family of flavonoids. For plant-derived CHIs, their evolutionary origin,[3] reaction mechanism,[4] and structures[5] have been extensively studied. More recently, it was found that CHI can also be found in some gammaproteobacteria and ascomycetes, but their physiological role remains to be determined.[6] One exception is the anaerobic gut microorganism *Eubacterium ramulus*. It has been reported that this strain is able to degrade a range of flavonoids, including naringenin-7-neohesperidoside, a glycosylated derivative of naringenin.[7] The authors suggested a possible degradation pathway, but did not present experimental verification of key intermediate steps. Furthermore, phloretin ($R^1 = OH$, $R^2 = H$; Scheme 1) is hydrolyzed in *E. ramulus* to 3-(4-hydroxyphenyl)propanoic acid and phloroglucinol, which is an undesirable pathway, as phloretin, as a degradation product of naringenin, is a very important flavor compound.[8] Although phloretin can in principle be chemically produced by reduction of the corresponding chalcone or by a Friedel–Crafts-type acylation of phenol with dihydrocinnamic acid,[9] these chemically synthesized products cannot be used as natural flavoring substances according to European flavor legislation. Therefore, an enzymatic or fermentative process for phloretin production would hold additional value for the flavor and fragrance



Naringenin: $R^1 = OH$, $R^2 = H$
Eriodictyol: $R^1 = OH$, $R^2 = OH$
Homoeriodictyol: $R^1 = OH$, $R^2 = OCH_3$

Scheme 1. Postulated pathway for the degradation of flavonoids. CHI = chalcone isomerase, ERED = enoate reductase. The equilibrium lies strongly on the side of flavanone formation.

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industry. Alternatively, glycosides of phloretin, such as phlorizin, can be obtained from *Malus* ssp.,[10] and are subsequently hydrolyzed to yield the aglycon phloretin.[11] However, this established process is time- and cost-intensive and also depends on the seasonal availability of the starting material. To avoid the seasonal impact and to simultaneously reduce production costs and waste streams, a biotechnological route for this important flavor compound is of high interest.

The cultivation of the strictly anaerobic *E. ramulus* DSM 16296 for the setup of an industrially useful bioprocess is an obstacle, as the growth is very slow, and whole-cell biotransformations lead to further degradation of phloretin.[12] These problems can be overcome by the heterologous expression of enzymes in microorganisms. This technique is already in use for the production of flavonoids from various substrates, such as L-phenylalanine, tyrosine, or cinnamic acid, but only for genes derived from plants.[13] In our present study, we came across several challenges, including the identification of the genes from the *E. ramulus* genome that encode the enzymes that are involved in the biocatalytic transformation of naringenin into phloretin, and the functional expression of the proteins in a recombinant host.

Herles and co-workers[14] determined a fragment of 15 amino acids at the N terminus of the CHI from *E. ramulus* DSM 16296 that is proposed to be involved in the conversion of naringenin into phloretin. This peptide sequence was used in the present study for the identification of the whole gene. Therefore, we sequenced the entire genome of *E. ramulus* DSM 16296 and used the resulting contigs to identify the encoding gene for the CHI. Only one hit was found, which aligned twelve consecutive residues of the published sequence directly after the starting methionine. This open reading frame (ORF; GenBank Accession Number: KF154734) corresponds to a protein of 32.5 kDa, which correlates well with the size that Herles and co-workers determined by SDS-PAGE for one subunit of the enzyme.[14] Interestingly, a BLAST[15] search did not identify any other CHIs from plant or other sources with a sequence identity of $\geq 10\,\%$ compared to this bacterial CHI; hence, this protein is unique (Supporting Information, Figure S1).

For the enoate reductase (ERED), no gene information was available. For this reason, we aligned 34 sequences of known EREDs and found a conserved motif that served as a basis to also identify the ERED-encoding gene in the *E. ramulus* DSM 16296 genome. Again, we found only one ORF (GenBank Accession Number: KF154735), its amino acid sequence shared low identity ($\leq 29\,\%$; Figure S2) with known EREDs.[16] However, all of these other EREDs have been only insufficiently characterized.

The genes that encode the bacterial CHI and ERED were cloned into common pET vectors, and functional expression in *E. coli* Rosetta was performed. We were pleased to find that the CHI from *E. ramulus* was expressed in soluble form under aerobic conditions in the *E. coli* Rosetta (DE3) strain (Figure S3). Slight modifications of the purification protocol described by Herles and co-workers[14] provided us with pure recombinant CHI for biochemical characterization and subsequent crystallographic analysis (Figure S4). The same purification protocol was also applied to the wild-type protein from *E. ramulus*; however, lower specific activities were found (Tables S1 and S2). The characterization of CHI revealed that the recombinant CHI maintains more than 90 % of its activity at pH 6.4–7.6 (Figure S5), whereas the optimal operational temperature is 45 °C (Figure S6). The recombinant CHI also exhibited satisfactory stability, maintaining more than 50 % of its initial activity after incubation at 41 °C for six hours (Figure S7).

The kinetic constants that were determined for the recombinant CHI underline the high activity of the enzyme (Figure S8). Although the K_m value of the recombinant bacterial CHI (36.9 μM) lies between those of the plant CHIs, which vary from 2 μM to 112 μM for the CHI from *Glycine max*[17] and *Medicago sativa*,[4] the turnover number is significantly higher. The k_{cat} values that have been reported in the literature for plant CHIs vary from 186 s^{-1}[4] to 833 s^{-1},[18] whereas a value of 4483 s^{-1} was determined for the recombinant CHI from *E. ramulus*. Hence, the recombinantly expressed bacterial CHI shows a catalytic efficiency of 1.2×10^8 M^{-1} s^{-1}, which is 75-times higher than the plant one from *Medicago sativa*, whose catalytic efficiency is 1.6×10^6 M^{-1} s^{-1}.[4] When we compared the recombinantly expressed CHI with the native CHI that was produced in *E. ramulus*, the kinetic values differed. Herles and co-workers determined the K_m to be 42.7 μM with a k_{cat} value of 2300 s^{-1}, which resulted in a catalytic efficiency of 0.5×10^8 M^{-1} s^{-1},[14] which is significantly lower than the one of the recombinant enzyme; this difference is mainly due to the different turnover numbers. The higher catalytic activity of the recombinant CHI can be attributed to the higher purity or to the formation of different multimers.

Analysis of the X-ray crystal structure (PDB code: 3zph) revealed that the recombinant CHI forms hexamers as trimers of three dimer units (Figure 1), whereas Herles and co-workers stated that only tetramers were observed in *E. ramulus*.[14] Regardless of this observation, the structure of this bacterial CHI differs significantly from those of plant CHIs; the protein–protein interactions of a dimer unit of the recombinant bacterial CHI are very strong owing to the incorporation of the C terminus of one protein chain into the second protein chain, whereas the plant enzymes mainly occur as monomers.[5] Moreover, in contrast to plant CHIs, an internal symmetry exists in the tertiary structure of a mono-

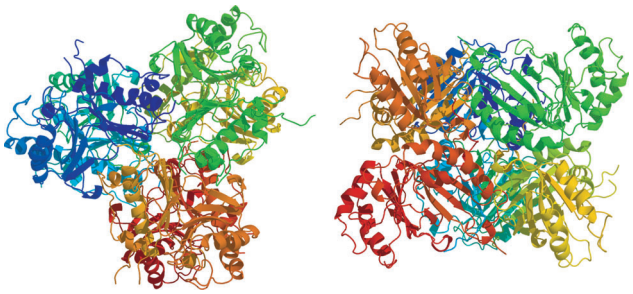

Figure 1. Crystal structure (left: top view; right: turned by 90°) of a hexamer with D_3 symmetry of the recombinant CHI from *E. ramulus*; PDB code: 3zph.

mer, as it can be divided into two domains with similar folds, which are rotated by 90° against each other.

In contrast to the expression of the CHI, expression of the ERED was more challenging. As the ERED gene with the native nucleotide sequence showed only a low expression level in *E. coli* Rosetta, a synthetic codon-optimized gene of ERED (sERED) was cloned into vector pET22b, which led to overexpression of soluble sERED at 20 °C under aerobic conditions. The protein had the expected molecular weight of 70 kDa, as determined by SDS-PAGE, which correlates well with the theoretical size of 75 kDa. Unfortunately, only low activity was observed for the conversion of the naringenin chalcone into phloretin. As the enzyme originates from a strictly anaerobic bacterium, we performed the expression under anaerobic conditions. Under nitrogen atmosphere, the highest expression level was observed at 25 °C. After ensuring that all steps (cell harvest, lysis, and biocatalysis) were performed under nitrogen atmosphere, significant enzymatic activity in the reduction of the chalcone to phloretin could be monitored. Furthermore, a crude cell extract that contained the sERED could be added to (purified) CHI, and conversion from naringenin into phloretin was observed. Pleasingly, the undesired enzymatic degradation of phloretin that was observed with *E. ramulus* did not take place with the *E. coli* system, thus enabling the synthesis of the target product.

The high oxygen sensitivity of the ERED from *E. ramulus* might be explained by its sequence similarity to a 2,4-dienoyl-CoA reductase and the anaerobic enoate reductase from *Clostridium* sp.[16a] These two enzymes are described as multi-domain proteins with a barrel domain that is related to the "Old Yellow Enzyme" with strong sequence conservation in a core region of approximately 40 amino acids. The second domain of this ERED is also redox-active and related to glutathione reductases with an iron–sulfur cluster, and could therefore be the oxygen-sensitive part of the protein. In this sequence, the four cysteines that are required for the iron–sulfur cluster were found in the typical motif $CXXCXXC(X)_{22}C$, a motif that also appears in the ERED from *E. ramulus*, starting at C361 (Figure S2).[14b] It should be noted that the sequence motif $GXGXXG(X)_{17}E$ for the NADH and FAD binding site in glutathione reductase[19] is found twice in the sequence of *E. ramulus* ERED.

To facilitate the industrial application of this bioprocess, we envisaged the simultaneous expression of both enzymes in an *E. coli* strain. To achieve this, the CHI gene was cloned with a ribosome binding site directly behind the sERED gene on the pET22b vector. With this new construct (pET22b_sERED/CHI), the soluble and active expression of both enzymes under anaerobic cultivation conditions was possible. Biocatalysis with the crude cell extract allowed the production of 50 μM phloretin after one hour under anaerobic conditions. Under aerobic conditions, both enzymes can be solubly expressed, although a significant amount of sERED is produced in its insoluble form (Figure S9). However, the amount of soluble sERED that was produced with this cultivation method is large enough to push the equilibrium towards the production of phloretin.

To evaluate the substrate specificity of this system and thus its versatility, *E. coli* BL21(DE3) cells with the construct pET22b_sERED/CHI were cultivated aerobically (see the Supporting Information), and biocatalytic transformations of three structurally similar flavanones were performed by whole cells under nitrogen atmosphere, as the aerobic process led to lower and reversible activity. High conversions were observed for naringenin, eriodictyol, and homoeriodictyol (Scheme 1) within a short period of time (Table 1). The

Table 1: Conversion [%] of flavanones into their respective dihydrochalcones.

Substrate	Reaction time		
	1 h	2 h	17 h
Naringenin	69 ± 1 %	86 ± 1 %	93 ± 1 %
Eriodictyol	46 ± 2 %	51 ± 4 %	72 ± 2 %
Homoeriodictyol	47 ± 3 %	52 ± 1 %	63 ± 2 %

hydroxyl group in the *para* position of the phenyl ring appears to be crucial for CHI activity; docking experiments of naringenin at the active site of the CHI showed that this hydroxyl group participates in a hydrogen-bonding network with Asp79 and Gln101, which is crucial for the right orientation of the substrate. On the other hand, these results show that the CHI seems to be tolerant towards substituents at the *meta* position of the phenol ring. Even though naringenin is the preferred substrate, as it only bears a hydrogen atom at this position, it seems that the active site of CHI can accommodate bulkier substituents, such as hydroxyl (eriodictyol) or methoxy (homoeriodictyol) groups.

In summary, the results of our experiments confirmed the metabolic pathway that was proposed by Herles and co-workers[14] for the degradation of flavonoids by the identification and successful recombinant expression of the chalcone isomerase and an enoate reductase from the anaerobic bacterium *Eubacterium ramulus*. The engineered *E. coli* strain that expresses both enzymes can be used for the conversion of several flavanones, which underlines the applicability of the biocatalytic system that was developed in this study.

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