

Schlussbericht zum Verwendungsnachweis

des Leibniz-Instituts für Pflanzenbiochemie (IPB)

zum Vorhaben:

FeruBase –

„Maßgeschneiderte Mikroorganismen für die Produktion von Ferulasäuren als Vorstufen hochwertiger Pflanzenprodukte“

Zuwendungsempfänger:	Leibniz-Institut für Pflanzenbiochemie
Förderkennzeichen:	031B0836B
Vorhabenbezeichnung:	"Maßgeschneiderte Mikroorganismen für die Produktion von Ferulasäuren als Vorstufen hochwertiger Pflanzenprodukte"
Laufzeit des Vorhabens:	01.02.2020 – 30.04.2023
Antragsteller:	Prof. Dr. Ludger Wessjohann
Mitarbeiter:	Dr. Martin Dippe Dr. Mohamed Nagia Dr. Miguel Fernandez-Niño Eric Pierschel Hannes Andrae

Das diesem Bericht zugrundeliegende Vorhaben wurde mit Mitteln des Bundesministeriums für Bildung und Forschung (BMBF) unter dem Förderkennzeichen 031B0836B gefördert. Die Verantwortung für den Inhalt dieser Veröffentlichung liegt beim Autor.

GEFÖRDERT VOM



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Teil I. Kurzbericht

The main aim of the project “Ferubase” was the construction of customized microorganisms for the production of high-value cinnamic acid derivatives, including certain taste modulators, antifungals and memory-enhancing natural products. The only source of the aforementioned compounds so far is the extraction from plants. However, such extraction processes are associated with a variety of problems. For example, the separation of the desired natural substance is complicated by the low concentration of the substances in the material, their environmental variation, and the occurrence of other isomeric compounds. Thus, biotechnological production represents a convenient and sustainable source for these metabolites.

The role of partner IPB was to provide suitable enzymes for the biocatalytic synthesis of these compounds and the respective genes for integration into the genomes of producer strains constructed by the partner Martin Luther University (MLU). The objective was accomplished through the implementation of various methodologies aimed at identifying enzymes suitable for large-scale production of specific metabolite groups for industrial purposes. The initial step involved a screening process to pinpoint the most appropriate enzymes. Subsequently, the identified candidates underwent optimization through rational design to tailor their functionality for specific tasks. These strategies led to the discovery of tailored transferase enzymes useful for the synthesis of specifically alkylated phenylpropanoids. Furthermore, through extensive genome and transcriptome mining, coupled with protein expression and enzymatic assays, novel acyltransferases were discovered in various plant species and characterized in detail in terms of their substrate and product spectrum. These enzymatic tools were subsequently shared with collaborators at MLU for integration into a bacterial strain engineered for the biotechnological production of certain biosynthetic precursors of the target compounds.

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Teil II. Ausführliche Darstellung

The overall goal of the cooperation project FeruBase was the development of a production strain for cinnamic acid derivatives. The focus of the project is directed toward health-promoting compounds and flavour profile modifiers. For the aim to be accomplished, an integration of the expertise from the Martin Luther University Halle-Wittenberg (MLU), the Leibniz Institute of Plant Biochemistry (IPB), and the Fraunhofer Center for Chemical-Biotechnological Processes (CBP) was required. The role of IPB is to provide suitable genes for biosynthetic enzymes for later integration into the genomes of producer strains developed at the MLU. The work plan at IPB was based on the following work packages (WP-I), which are thematically oriented to the enzymes to be developed:

AP E.1: Identification / Design of O-methyltransferases for cinnamate synthesis (months 1-24)

O-methyltransferases (OMTs) catalyze the transfer of a methyl group from a methyl donor to an oxygen of a hydroxy or carboxy group. We tested a set of plant-derived OMTs available at the IPB which are known to have regioselective methylation of a catecholic systems. For methylation in the meta-position of simple phenylpropanoids (cinnamates) and the more complex flavonoids, the enzyme *PFOMT* from the ice plant *Mesembryanthemum crystallinum* proved to be an efficient tool. Characterization of this enzyme, which was summarized in a publication (Dippe et al. (2022) ChemCatChem 14, e202200511), showed a high conversion rate for a range of different catechols with no para-activity. To access regioisomers with *para*-methylation, other plant enzymes had to be developed. This process involved engineering of candidates by a rational engineering strategy. Similar to the engineering of *PFOMT* to customize its substrate specificity (Dippe et al. (2022) ChemCatChem 14, e202200511), and enzyme from a plant alkaloid biosynthesis route was iteratively improved from an initially very low to good *para*-selectivity (78 %). With these results, the milestone IPB-ME-2 was accomplished.

AP E.2: Design of chalcone synthases for the production of flavonoids (months 1-12)

Flavonoids can be formed from cinnamates via a two-step pathway. First, a cinnamic acid-coenzyme A (CoA) ligase enzyme activates the corresponding acid to its CoA thioester. Secondly, a chalcone synthase condenses three molecules of malonyl CoA on the activated cinnamoyl derivative. To achieve the aim of this work package, we developed a p-coumaroyl-

CoA ligase known for its promiscuity toward various substituted hydroxy- and methoxycinnamic acids to form the respective thioesters in high yield (Dippe et al. (2019) Adv. Synth. Catal. 361, 5363). For the second reaction step, a plant chalcone synthase with high activity was selected as a model enzyme for further engineering to catalyze the conversion of certain CoA thioesters into the desired flavanones. Unlike typical chalcone synthases, this enzyme proved to be is well expressed in *E. coli* and highly active in the conversion of the native substrate p-coumaroyl-CoA. Rational design of the enzyme started with constructing a 3D model of its active site. The constructed model was then used to select residues that can be exchanged in order to raise activity on a set of CoA-conjugated cinnamic substrates. As a number of the different variants produced during this process showed conversion of p-coumaroyl-CoA but also non-native conjugates, the milestone IPB-ME-2 was thus successfully fulfilled.

AP E.3: Identification / Design of transferases for the production of cinnamate conjugates (months 1-24)

Amides and esters of cinnamates are involved in plant's defense against pathogens such as the oomycete *Phytophthora infestans*, the causative agent of the devastating late blight disease in Solanaceae, and are interesting for industrial applications due to their antioxidant and physiological (taste-modulating, memory-enhancing) effects. The formation of these valuable compounds requires the identification and design of promiscuous acyltransferases. Thus, a plant defensive acyltransferase found in a mining approach was characterized in detail to judge its applicability in whole-cell biocatalysis. Therefore, the conversion of seventeen structurally diverse cinnamic, benzoic, and aliphatic CoA esters to phenolic amides was tested using a recombinant enzyme heterologously expressed in *E. coli*. In addition, the acceptance of twelve structurally diverse amines was probed. HPLC and HPLC-MS analysis of the enzymatic assays showed exceptional promiscuity, as all the tested CoA thioesters and even nine of the amine acceptors were converted to the corresponding amides. Based on these results, enzymatic reactions were scaled to test the antifungal activity of the formed amides against *P. infestans*. Therefore, an anti-oomycete assay was established, and eight enzymatically formed amides were tested against the potato pathogen. These results show that selected amides have a pronounced anti-oomycete activity. In summary, a highly active and promiscuous amide synthase was made available for the consortium.

Certain cinnamic acid esters proved to have strong memory-enhancing activities (Michels et al. (2018) *Sci. Adv.* 4, eaat6994). Their biosynthetic production was first probed using a known BAHD-type synthase and yet undescribed candidates identified in transcriptomes and genomes of plants which are producers of cinnamate esters. In the latter mining, 18 full-length genes were identified and tested via activity profiling. Six of these genes could be expressed successfully in *E. coli*, from which three enzymes were active in in vitro reactions. Its enzymatic products were investigated by GC-MS, HPLC, and UPLC-MS, showing the promiscuity of all the tested enzymes that accepted all the tested acyl donors and acceptors. In addition, we proved the formation of new-to-nature esters derived from non-natural CoA esters. By building up this toolbox of promiscuous acyltransferases, milestone IPB-ME-3 was fulfilled.

AP E.4: Design and testing of reaction cascades (months 10-33)

A highly active acyltransferase enzyme identified in AP E.3 was selected to perform an in vitro one-pot cascade reaction by coupling to an upstream ligase for the production of a cinnamic amide in a proof-of-concept study. As a prior step to calculate the optimum amount of each enzyme required for the reaction, the specific activity of both enzymes under the same condition was determined. Therefore, the concentration of free CoA (as a remaining substrate in the case of the CoA ligase or as a product in the case of the subsequent transferase reaction) was determined spectrophotometrically using Ellman's reagent by comparison to a standard curve of free CoA (Dippe et al. (2019) *Adv. Synth. Catal.* 361, 5363). The cascade reaction for the formation of the amide was then performed under saturating conditions of equimolar concentrations of the reactants (i.e. the used cinnamate, amine and CoA). Two sets of negative controls were performed in parallel. HPLC analysis of the enzymatic incubations showed a peak of the expected amide with conversion rates up to 80 %, proving that the developed cascade is functional.

AP E.5: Integration of the reaction cascade into a producer strain

The compatibility the reaction cascade proven during the work in AP E.4 was the basis for a transfer into a cellular system. Thus, plasmids harbouring the genes encoding both involved enzymes were handed to the project partner MLU for integration in a cinnamate producer strain. Thus, IPB work share in milestone IPB-ME-4 was fulfilled as required.