CHEMCATCHEM

DOI: 10.1002/cctc.201000429

Development of a Whole Cell Biocatalyst for the Efficient Prenylation of Indole Derivatives by Autodisplay of the Aromatic Prenyltransferase FgaPT2

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The following study depicts the development of a whole cell biocatalyst for the prenylation of indole derivatives. For this purpose the prenyltransferase FgaPT2 from *Aspergillus fumigatus* was displayed on the surface of *Escherichia coli* cells by using Autodisplay. The presence of the prenyltransferase in the outer membrane was detected by using SDS-PAGE and Western Blot after the proteins of the outer membrane were isolated. The orientation of the prenyltransferase towards the outside of the cells was investigated by accessibility testing with externally added proteases. The FgaPT2 whole cell biocatalyst converted up to 250 μM of indole-3-propionic acid, approximately 25% of the substrate used in the assay (100 μL sample, $OD_{578}=40$). Another indole substrate, L-β-homotryptophan was also investigated and a conversion of 13% was determined. By optimizing the assay conditions the conversion rate could be raised to approximately 30% of indole-3-propionic acid during a 24 h incubation time at 20 °C. The whole cell biocatalyst endured a storage period of one month at 8 °C without any detectable loss in activity. Reusability was confirmed by recycling the biocatalyst. After three cycles of consecutive use, the whole cell biocatalyst retained a conversion rate of 46% of indole-3-propionic acid and 23% of L-β-homotryptophan after the third cycle.

Introduction

Prenyltransferases catalyze the transfer of an activated isoprenoid moiety derived from allylic isoprenyl diphosphates [e.g., dimethylallyl diphosphate (C_5), geranyl diphosphate (C_{10}) or farnesyl diphosphate (C_{15})] onto a nucleophilic acceptor molecule. They are subdivided into three groups being isoprenylpyrophosphate synthases, protein prenyltransferases and aromatic prenyltransferases.[1] Isoprenylpyrophosphate synthases catalyze the chain elongation of allylic pyrophosphate substrates with isopentenyl pyrophosphate. The resulting isoprenoids are precursors for a large variety of natural products for example, terpenes, and steroids.[2–3] Protein prenyltransferases catalyze the transfer of a prenyl moiety (C_{15} or C_{20}) onto protein or peptide substrates. This posttranslational modification is vital for the function of signal transducing proteins, such as Ras or other G-proteins.[4–5]

Aromatic prenyltransferases attach isoprenoid moieties to aromatic acceptor molecules. Based upon this prenylation reaction, which is often the rate limiting step in the respective pathway, many different primary and secondary metabolites are generated in plants, fungi, and bacteria.[6–7] It has been shown that this prenylation step enhances the pharmacological activities of some flavonoids compared to their non-prenylated precursors.[8–9] This effect is presumably caused by an increase in the affinity for biological membranes and improved interactions with cellular targets.[10]

Another class of prenylated compounds from *Aspergillus fumigatus* (*A. fumigatus*), tryptophan derived cyclic dipeptides, exhibited enormous cytotoxic effects.[11] Prenylated products also function as precursors or intermediates for secondary metabolites.[7] For example the first step in ergot alkaloid synthesis is the prenylation of tryptophan.[12] Because of their low substrate specificity, aromatic prenyltransferases can be regarded as interesting biocatalysts for the synthesis of new pharmacologically active compounds or intermediates.[13] FgaPT2 is an aromatic prenyltransferase from *A. fumigatus*. It is a soluble homodimeric protein with a subunit size of 52 kDa.[14] It catalyzes the first step in ergot alkaloid synthesis in *A. fumigatus* by transferring an activated dimethylallyl group to tryptophan and, therefore, can be more specifically described as a dimethylallyltryptophan synthase.[12]

The gene encoding for the dimethylallyltryptophan synthase FgaPT2 used in this study was originally identified in the genome sequence of *A. fumigatus*, heterologously expressed in *Saccharomyces cerevisiae*, and therein isolated and character-

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cctc.201000429.

ized.[15] The FgaPT2 has also been expressed in *Escherichia coli* (*E. coli*), thus far, and prenylation of different indole derivatives has been investigated.[16] Similar to most of the aromatic prenyltransferases, which show a low substrate specificity,[7,13] FgaPT2 also exhibits substrate promiscuity and accepts 17 different indole derivatives.[16] A recent study suggests that also tryptophan containing dipeptides are accepted as substrates by FgaPT2.[17] It thus represents a promising biocatalytic tool in enzymatic synthesis of active indole or indole-dipeptide derivatives.[7,13]

One way to establish an efficient biocatalytic process is to use whole cell biocatalysts constructed using surface display. Enzymes displayed on the cell surface of, for example, gram negative bacteria, are immobilized and thus more stable.[18] Presentation of the enzyme on the surface enables the direct contact between substrate and enzyme, making the membrane passage of the substrate unnecessary. Coupling the enzyme to an insoluble and rather large particle like an *E. coli* cell, furthermore allows the time- and cost-effective recovery of the biocatalyst. Maintaining the biocatalyst is achieved by simply cultivating the *E. coli* cells.

A proven method for the development of surface engineered whole cell biocatalysts is Autodisplay. The Autodisplay system allows the display of different types of proteins on the surface of *E. coli*.[19] These proteins are integrated as a passenger in a precursor protein consisting of a signal peptide, which facilitates the transport across the inner membrane, and a C-terminal β-barrel structure, which enables the translocation across the outer membrane. The passenger and β-barrel are connected by a linker peptide, assuring the complete display of the protein of interest (Figure 1).[20] This translocation subunit consisting of linker and β-barrel subunit is derived from the AIDA-I (adhesine involved in diffuse adherence) autotransporter protein of *E. coli*.[21] Since the β-barrels are motile in the outer membrane, multimeric proteins displayed with this technique show the spontaneous aggregation of passengers to form dimers or even higher ordered oligomers.[22–24] Examples of functional enzymes expressed using Autodisplay for functional whole cell biocatalysis include an esterase, a sorbitol dehydrogenase, and a nitrilase.[23–25]

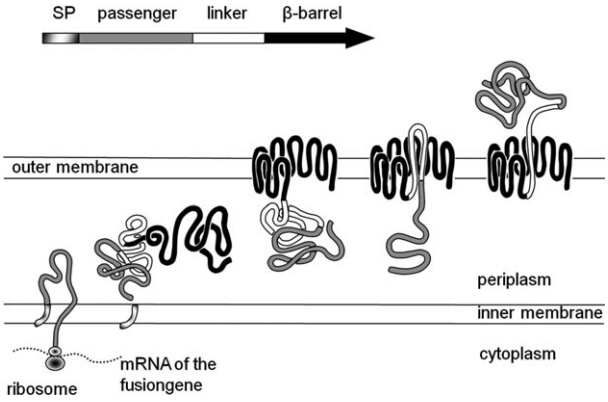

Figure 1. Translocation mechanism of autotransporter proteins. The N-terminal signal peptide facilitates the transport across the inner membrane and is then cut off by using periplasmic signal peptidases. The C-terminal part forms a β-barrel structure inside the outer membrane, through which the passenger is translocated to the surface by using the linker.

In this study, employing this variable and powerful tool for the surface display of active prenyltransferase FgaPT2 we managed to develop a stable and reusable whole cell biocatalyst for the effective prenylation of indole derivatives.

Results

Construction of a dimethylallyl-transferase- autotransporter fusion protein

The *fgaPT2* gene was amplified by polymerase chain reaction (PCR) from plasmid pIU18.[16] The PCR primers added an XhoI site at the 5′ end and a KpnI site at the 3′ end of *fgaPT2*. The PCR fragment was inserted into pCR4-TOPO-vector and recleaved with XhoI and KpnI. The restriction fragment was ligated into pET-Adx04, which had also been cleaved with the same enzymes. Insertion of the *fgaPT2* PCR fragment resulted in plasmid pEK004 which encodes an artificial fusion protein composed of an N-terminal signal sequence derived from choleratoxin β- subunit, FgaPT2 as a passenger and the C-terminal AIDA-I autotransporter region (Figure 2). Within this autotransporter a linker and a β-barrel structure cause the translocation across the outer membrane (Figure 1). Plasmid pEK004 was transformed into *E. coli* BL21(DE3). Expression of the autotransporter fusion protein was induced by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG), to a final concentration of 1 mM, for one hour at 30 °C at 200 rpm. Isolates of outer membrane proteins were analyzed by SDS-PAGE. A basal expression of fusion protein occurred even in the absence of IPTG (Figure 3 A and B, lane 1). Presumably, this was because of the leakiness of the *lac*UV promoter.[26]

Surface display of FgaPT2

To prove whether surface exposure occurred effectively, the accessibility of the fusion protein for proteases was tested. To do this, protein expression was induced and whole cells were subsequently treated with Proteinase K. As proteases are too large to cross the outer membrane, proteins can only be digested by proteases when exposed at the surface. Treatment of whole cells with Proteinase K resulted in digestion of the fusion protein (Figure 3). To demonstrate whether the outer membrane stays intact during the proteinase accessibility test, OmpA can be used as an integrity reporter for the outer membrane. The C-terminal part of OmpA directs to the periplasmic space between the inner and outer membrane. The N-terminal part builds a β-barrel structure inside the outer membrane, but does not point to the outside.[27] Thus OmpA is only sensitive against digestion from the periplasmic side, but not from the outside and therefore indicates membrane integrity. If a protease is added and only the fusion protein, but not OmpA is digested, integrity of the cell is assured and the fusion protein can be regarded as surface exposed. Since OmpA was not digested by Proteinase K during our experiment, the accessibility test provided strong proof of surface exposure (Figure 3 A,

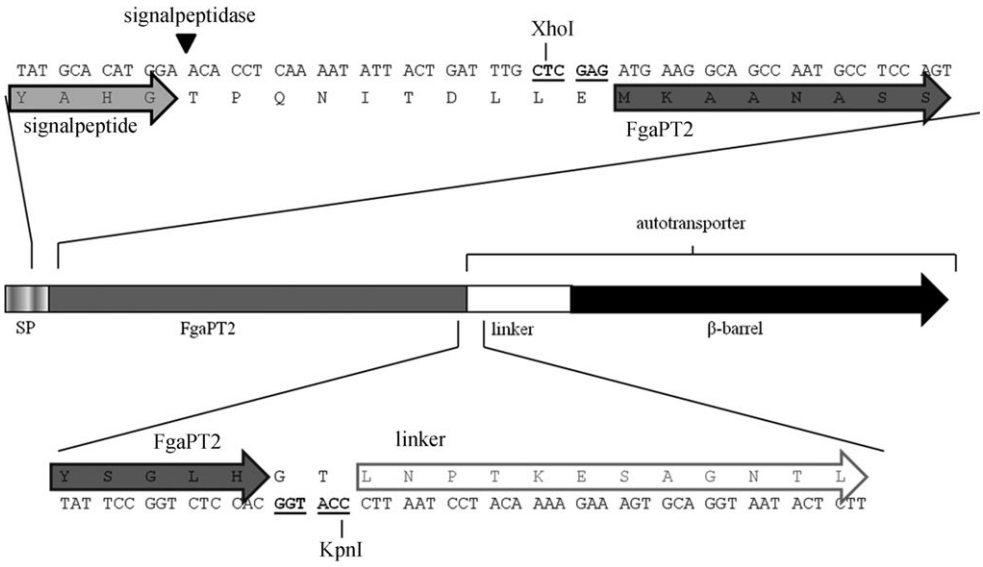

Figure 2. Structure of the precursor protein FgaPT2-AT encoded by using pEK004. The environment of the fusion sites is given as nucleotide and amino acid sequences. The restriction sites used for cloning are underlined. The N- and C-terminal sequences of the FgaPT2 passenger are indicated by using a grey arrow. The C-terminus of the signal peptide is represented by using a light grey arrow whereas the white arrow depicts the N-terminus of the linker region.

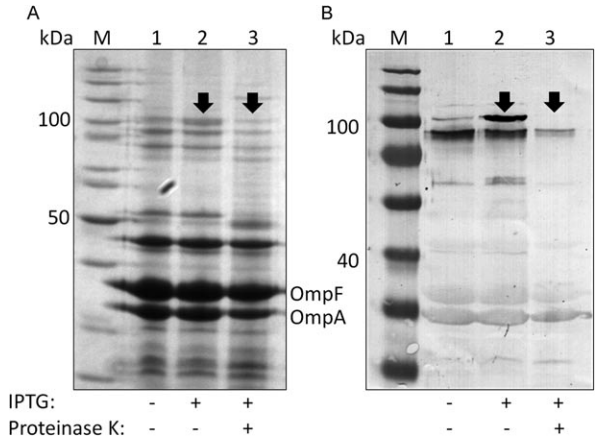

Figure 3. Surface display of FgaPT2-AT-fusion protein. A) SDS-PAGE and B) Western Blot analysis of the outer-membrane protein preparation from *E. coli* BL21(DE3) pEK004 (lane 1–3). Molecular weight markers are indicated on the left hand side. M: protein marker; IPTG: protein expression was induced by adding IPTG (final concentration: 1 mM; Proteinase K: whole cells were treated with Proteinase K; OmpA/OmpF: natural *E. coli* outer-membrane proteins. The FgaPT2-AT fusion protein is indicated by using black arrows.

lane 3). We did notice a slight difference in intensity of the protein bands of OmpA as well as of OmpF (lane 3) compared to the non digested sample (lane 2). This might be due to a minor difference in protein concentration. The Western Blot prepared from the same samples (Figure 3B) shows a clear decrease in intensity of the fusion protein attributed to the Proteinase K treatment.

Enzyme activity of surface displayed FgaPT2

After confirming the surface exposure of FgaPT2, the cells displaying FgaPT2 were tested for enzymatic activity. Cells were prepared as described above and conversion of 1 mM indole-3-propionic acid and L-β-homotryptophan was performed for 24 h in 100 μL assay samples. Reaction products were analyzed by HPLC. Host cells *E. coli* BL21(DE3) without a plasmid, used as a negative control, did not show dimethylallyltransferase activity (Figure 4A). Cells harboring the plasmid encoding for the autotransporter fusion protein showed dimethylallyltransferase activity even without induction of protein expression (Figure 4B). This was presumably due to a leakiness of the *lac*UV promoter, a common effect that causes a basal expression of active fusion protein. After induction of protein expression, cells displaying the fusion protein clearly showed higher activi-

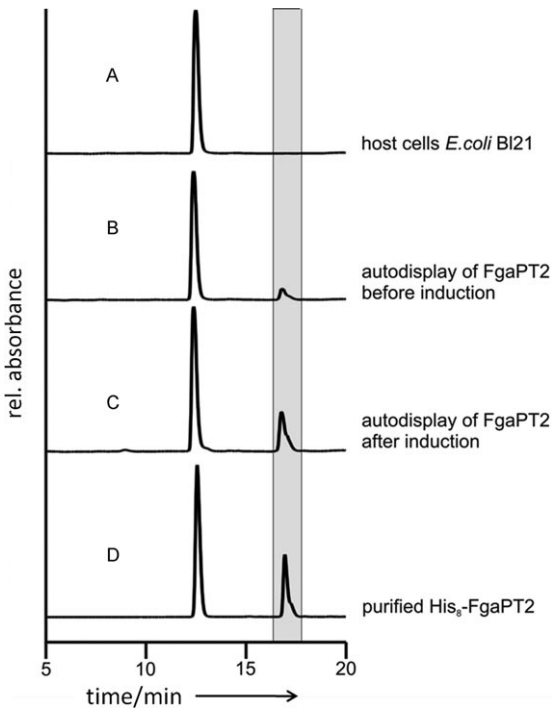

Figure 4. HPLC chromatograms of the assays using the FgaPT2 whole-cell biocatalyst B) before and C) after induction. Host cells (A) and the purified enzyme His8-FgaPT2 (D) were used as controls. HPLC chromatography was performed on a RP18 column at a flow rate of 1 mL min^{-1}. The shaded area emphasizes the product peak. The substrate (indole-3-propionic-acid) elutes at a retention time of approximately 12–13 min.

ty (Figure 4C). The retention time of reaction products of conversion with whole cells displaying the fusion protein on the surface corresponded very well to the retention time of reaction product yielded with purified enzyme His_8-FgaPT2, which was used as a positive control (Figure 4D).

Conversion rates were then calculated, giving 100 μM of 4-dimethylallyl (DMA)-indole-3-propionic acid, respectively 64 μM of 4-DMA-β-homotryptophan were formed in samples before induction of protein expression (Figure 5C). Samples after induction of protein expression contained 254 μM of 4-DMA-indole-3-propionic acid and approximately 133 μM formation of 4-DMA-β-homotryptophan (Figure 5C).

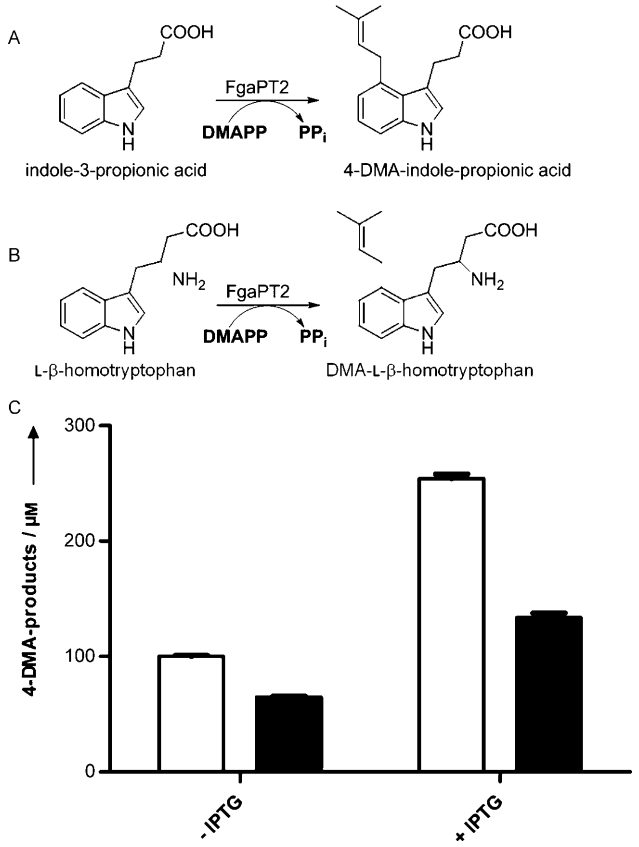

Figure 5. Prenylation of A) indole-3-propionic acid and B) L-β-homotryptophan at *C*4, resulting in 4-dimethylallyl-propionic acid and 4-dimethylallyl-tryptophan, respectively. C) Enzymatic activity of whole cells expressing FgaPT2. Conversion of indole-3-propionic acid (white columns) and L-β-homotryptophan (black columns) was analyzed before and after induction by using IPTG. The reaction was run in a sample volume of 100 μL at an $OD_{578} = 40$ for 24 h. Reaction products were determined by applying HPLC to the supernatant after removing the cells in a centrifuge. Mean values of three independent measurements are shown; standard deviations are included as error bars.

Identification of the enzymatic products by LC-MS:

The corresponding mass of the enzymatic products yielded with the whole cell biocatalyst was determined by mass spectrometry. The obtained fragmentation pattern (Table S1 in the Supporting Information) was in good accordance with the data for 4-DMA-indole-3-propionic acid and 4-DMA-β-homotryptophan published previously.[16] It has already been shown in a previous study, that the prenylation by FgaPT2 takes place regiospecifically at position 4 of the indole ring.[15–16]

Influence of incubation temperature and DMAPP concentrations on the enzymatic reaction

To optimize enzymatic activity of the surface displayed FgaPT2 incubation temperature and concentration of dimethylallyl pyrophosphate (DMAPP) cosubstrate were modified. The highest conversion of substrate was achieved at 20 °C (Figure 6). Additionally, if the concentration of cosubstrate was raised from 1 mM to 5 mM and 10 mM the conversion rate at 20 °C increased from 20% to almost 30%. Conversely, incubation at 35 °C achieved less than 10% of substrate conversion. At these conditions increasing concentrations of cosubstrate did not result in higher conversion rates. We attributed this to the fact that temperature and not concentration of cosubstrate was the limiting factor. This effect could be observed when conversion was performed at 25 °C and 30 °C. Raising DMAPP concentrations from 1 mM up to 5 mM caused a further increase in substrate conversion from 8 to 13% at 25 °C and from about 15 to 20% at 30 °C, respectively. At these temperatures enzymatic activity of surface displayed FgaPT2 is higher, but 5 mM of cosubstrate was sufficient to sustain the enzymatic reaction for 24 h. In summary, the optimum enzymatic activity of surface displayed FgaPT2 was at 20 °C incubation temperature and 10 mM cosubstrate concentration.

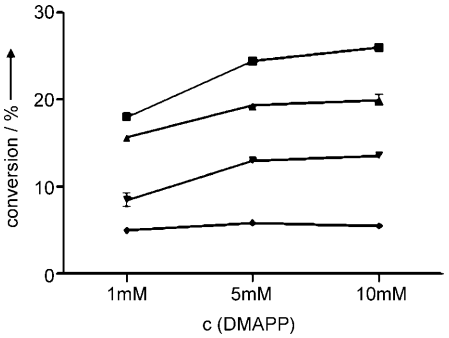

Figure 6. Dependence of product formation on different assay conditions. FgaPT2 activity of whole cells was analyzed at different temperatures and different concentrations of DMAPP. Conversion rates of indole-3-propionic acid are shown for 20 °C (■), 25 °C (▲), 30 °C (▼), and 35 °C (◆). The reaction product was determined by using HPLC. Mean values and standard deviations of three separate runs are presented.

There is a notable difference in the turbidity values of *E. coli* cell suspensions of high densities, when measured at different temperatures. It can lead up to 19% lower values at 20 °C in comparison to those measured at 35 °C (see the Supporting Information). This decrease in measured turbidity at lower temperatures, however, cannot account for the massive increase in activity observed under incubation of the FgaPT2-whole cell biocatalysts at lower temperatures. Activity at 20 °C was 5 fold of the activity at 35 °C.

Reusability of the FgaPT2 whole cell biocatalyst

One of the advantages of whole cell biocatalysts is their simple availability and the possibility to easily recover the cells from the reaction mixture. Therefore, the cells need to be sufficiently stable to endure the recovery treatment. Hence the reusability of the FgaPT2 whole cell biocatalyst should be analyzed in three consecutive activity test cycles. Assays were performed with 1 mM indole-3-propionic acid or L-β-homotryptophan and 1 mM of DMAPP cosubstrate at 20 °C. After 24 h of incubation, the cells were removed by centrifugation. The supernatant was analyzed by HPLC. The cells were resuspended in buffer, substrates were added and the assays were again incubated at 20 °C. Procedure was repeated every 24 h for 3 days. In comparison to the first day, the enzyme retained 46% and 23% of its activity in the production of 4-DMA-indole-propionic-acid and DMA-β-homotryptophan, respectively (Figure 7). Re-growing or autolysis of cells throughout the different reaction cycles was not monitored in the present experiments. According to our results with other whole cell biocatalysts obtained by applying the Autodisplay system,[24] and in comparison with this study, there is no significant re-growth or autolysis detectable.

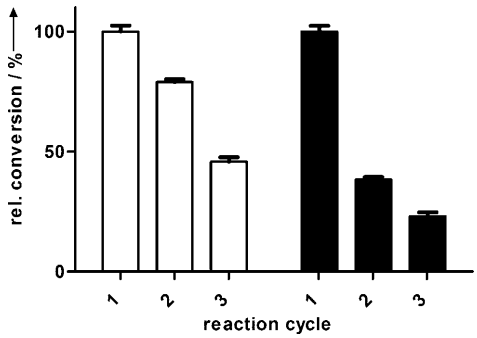

Figure 7. Reusability of the whole-cell biocatalyst for each cycle of 3 reaction cycles. Conversion of indole-3-propionic-acid (white columns) and L-β-homotryptophan (black columns) was tested and determined by using HPLC after 24 h of incubation for each cycle. The relative rate of conversion is presented by defining the first conversion as 100%. Mean values of three independent measurements are shown; standard deviations are included as error bars.

Long term stability of stored whole cell biocatalysts

To determine long term stability of the whole cell biocatalyst, a suspension of induced cells in buffer ($OD_{578} = 50$) without any conservation additives was stored at 4 °C for 1 month. To avoid sedimentation of cells, the suspension was mixed every 24 h by inversion of the tubes several times. Before and after one month of storage, activity was determined. Compared to the conversion of substrate measured before storage, no decrease in enzymatic activity of stored cells was observed. The number of viable cells in the freshly prepared and stored samples was also determined. Although there was no decrease in activity, the number of viable cells in one assay sample differed 10 fold from 4.2 million cells per 100 μL in the fresh sample to 400 000 cells per 100 μL in the stored one.

Efficiency of the whole cell biocatalyst

To examine the efficiency of the whole cell biocatalyst, the number of enzyme molecules displayed on a single cell by using Autodisplay was calculated first. For this purpose, outer membrane proteins of such cells that had been used for enzyme activity measurement were prepared and analyzed by SDS-PAGE (see the Supporting Information). After staining with Commassie Brilliant Blue, the intensity of the band of the FgaPT2 fusion protein was compared to the intensity of the OmpA band by using the GelPro Analyzer software (Media Cybernetics, Bethesda, USA). OmpA is known to be expressed at constant levels of about 1×10^5 molecules per single cell of *E. coli*.[28] According to GelPro Analyzer, the intensity of the FgaPT2 fusion protein band was three times of the intensity of the OmpA band. The fusion protein has a calculated molecular mass of 106 kDa, whereas OmpA has a molecular mass of 35 kDa. In conclusion, the threefold higher intensity of the FgaPT2 fusion protein band, when compared to the OmpA band, must a result a threefold higher molecular mass of an identical number of molecules. Hence, the number of surface displayed FgaPT2-fusion proteins is supposed to be approximately 1×10^5 monomers per single cell, and —as functional FgaPT2 has been shown to be a dimmer—5×10^4 functional dimers per single cell.

The number of viable cells displaying FgaPT2 was determined to be 4×10^6 per assay sample by plating out adequate dilutions on agar plates. In consequence the entire number of surface displayed enzyme molecules in an assay sample could be calculated and turned out to be in the range of about 2×10^{11} (Table 1). The number of purified His_8-FgaPT2 enzyme molecules in the purified enzyme sample used as a control was calculated on the basis of the molecular mass of the enzyme and the added amount to an assay sample using the Avogadro constant. Taking into account that dimers need to be formed, the number of active molecules purified enzyme sample was estimated to be around approximately 6×10^{12}. This means that the sample with purified His_8-FgaPT2 contained 30 times more enzyme molecules than the sample containing the whole cell biocatalyst. Based on these findings a direct deter-

Table 1. Efficiency of the FgaPT2-whole cell biocatalyst compared to purified enzyme.

	Enzyme molecules per sample	Substrate conversion [mM][a]	Relative conversion per enzyme molecule [fmol]
purified enzyme	6×10^{12}	0.34	5.66×10^{-6}
whole cell biocatalyst	2×10^{11}	0.254	1.27×10^{-4}

[a] 1 mM start concentration of indole-3-propionic acid.

mination of efficiency of the FgaPT2 whole cell biocatalyst was possible. We then compared the conversion rates of indole-3-propionic acid in both assays. Although the number of enzyme molecules was lower, the whole cell biocatalyst reached nearly 75% of the conversion yielded by the purified enzyme during the same reaction time. Both conversions, with whole cell biocatalyst and purified enzyme respectively, were performed for 24 h. By determining the relative conversion rates, it turned out, that the surface displayed enzymes converted twenty times more substrate than the purified enzyme molecules (Table 1). This could be an indication for an improved stability of the surface displayed FgaPT2. For the purified enzyme a conversion of 32% of the used substrate indole-3-propionic acid within one hour of reaction time is reported.[16] Increasing the reaction time to 24 h did not greatly improve the yield of converted substrate (34%), suggesting that the enzyme is instable and rapidly loses activity. In comparison the present study shows that an efficient and obviously more stable application of FgaPT2 could be achieved by Autodisplay of FgaPT2 and employment of whole cells for substrate conversion.

Discussion

Within this study we used Autodisplay for the development of a functional whole cell biocatalyst for the synthesis of prenylated indole derivatives. The attachment of a prenyl moiety to an aromatic acceptor often increases the biological effect of the molecule. C8-prenylation of the flavonoids apigenin and liquiritigenin dramatically enhances their cytotoxic effect compared to their non-prenylated precursors.[9] Tryprostatins, prenylated derivatives of *cyclo*-L-Trp-L-Pro derived from *A. fumigatus*, showed cell cycle inhibiting effects.[29] One diastereomer of tryprostatin B exhibited an even more potent cytotoxicity profile than etoposide when it was tested in human prostate, lung, and breast cancer cell lines.[30] Hence the prenylation step is of tremendous importance not only during the biosynthesis of cytotoxically active second metabolites in plants and fungi but most likely also in a chemoenzymatic synthesis of possible anti tumor agents. Even though the total synthesis of tryprostatin A and B has already been demonstrated,[31] employment of a biocatalyst for prenylation offers the advantages of lower energy consumption, regiospecific attack and less bothersome by-products, since the prenylation step in the chemical synthesis of tryprostatins needs to take place under the use of isoprenylbromide and at −78 °C. The biocatalyst also offers the opportunity for synthesis of a variety of active compounds and intermediates, because aromatic prenyltransferases show a low substrate specificity and are thus able to accept a rather broad diversity of substrates.[7,13]

The application of surface display systems, such as Autodisplay, for the development of whole cell biocatalysts comprises many advantages compared with conventional whole cell biocatalysts, for which the enzyme of interest resides within the cell cytoplasm. Firstly, transport across membranes is not necessary for substrates or products;[18] secondly, product and catalyst can be easily recovered by removing the cells from the reaction; thirdly, it is possible to avoid the formation of by-products or metabolism of the substrate or desired product inside the cell, which is often a problem in whole cell biocatalysis.[32–33]

When incubated with indole-3-propionic acid as a substrate for 24 h, cells displaying FgaPT2 on their surface showed a conversion of about 25% of the deployed substrate after protein expression was induced by adding IPTG. In cells which were not treated with IPTG, a conversion of 10% of indole-3-propionic acid was measured. It is a reasonable assumption that this activity is caused by a basal expression of the desired protein, caused by leakiness of the *lac*UV promoter that controls T7 polymerase expression in the applied *E. coli* strain BL21(DE3).[34] The conversion of a second indole substrate, L-β-homotryptophan, within 24 h by the whole cell biocatalyst was determined to be 13% after induction and 6% before induction. It was not possible to determine conversion of the native substrate tryptophan, as it is already completely converted into another not prenylated product by the host cells *E. coli* BL21(DE3) and some other expression strains that we tested (*E. coli* UT5600(DE3), data not shown).

The FgaPT2 whole cell biocatalyst showed the highest activity (30% of substrate converted) at 20 °C and addition of 10 mM of DMAPP cosubstrate. These can be considered as very mild conditions, because a high conversion rate was yielded even at room temperature instead of 30 °C, which was the temperature optimum for the purified enzyme.[15] Furthermore, the functional expression of FgaPT2 provides further evidence for the motility of the β-barrels in the outer membrane. As FgaPT2 is supposed to be active only in a dimeric state,[14] our results suggest that dimerization occurred on the outer membrane. This effect of spontaneous dimerization has already been shown for the Autodisplay of bovine adrenodoxin[22] and sorbitol dehydrogenase.[23] Even higher aggregates could be functionally expressed by Autodisplay, as shown for a multihomomeric nitrilase from *Alcaligenes faecalis*.[24]

In applying the FgaPT2-whole cell biocatalyst in three consecutive activity assays with recovery of the catalyst by means of centrifugation, the whole cell biocatalyst was reusable and after three cycles retained 46% and 23% of its activity towards indole-3-propionic acid and L-β-homotryptophan, respectively. The loss of activity may be attributed to a loss of cells during the centrifugation and resuspension steps. It is also possible that a certain amount of surface displayed protein suffers from abrasion during the treatment since a relatively high optical density ($OD_{578} = 40$) was used, possibly resulting in mechanical stress of the surface displayed proteins.

The storage of cell samples for 1 month at 8 °C was also possible without any loss of activity. After one month of storage the number of viable cells decreased to 10% of the original sample, however, the stored cells showed the same conversion rate as a fresh sample. The herein tested viability only describes the cells ability to reproduce. The measured loss of viability therefore is not equivalent with death or lysis of cells. As long as the cell or the outer membrane remains intact respectively, the surface displayed enzymes still can be active. Storage at −20 °C or −70 °C was not tested but also is theoretically possible. Storage stability at −70 °C was proven to be possible

for a whole cell biocatalyst displaying a nitrilase from *A. faecalis* by means of Autodisplay.[24] In this case the addition of 20% glycerol to the suspension of cells was necessary to prevent a loss of cells. For the depicted storage experiment with the FgaPT2-whole cell biocatalyst at 4 °C the addition of such conservation additives was not necessary.

When compared to conversions of indole-3-propionic acid by the purified enzyme, the whole cell biocatalyst showed its effectiveness. The conversion of indole-3-propionic acid of the purified enzyme was 34% in 24 h. During one hour of reaction time 32% of indole-3-propionic acid were converted, whereas the native substrate tryptophan was converted completely.[16] Regarding the number of enzymes accomplishing this conversion in an assay sample (Table 1) there was 30 times more molecules of purified enzyme within one sample than enzyme molecules on the surface of the whole cell biocatalyst. Taking into account that there was no further increase in substrate conversion by the purified enzyme when the reaction time was extended, it can be assumed that the enzyme is instable and rapidly loses its activity. So with a substrate conversion of 25% by the whole cell biocatalyst, nearly 75% of conversion rate yielded by the purified enzymes were reached with less molecules of enzymes, suggesting that Autodisplay increases the stability of FgaPT2.

In this work we again demonstrated the capability of Autodisplay for the development of functional whole cell biocatalysts. The surface displayed enzymes showed excellent performance towards the tested substrates and a high efficiency of conversion under very mild conditions. By immobilizing the enzyme in the outer membrane of *E. coli* its stability was improved enormously. As the purified enzyme is reported to act on the dipeptide precursor molecules of tryprostatins,[17] these molecules can be tested as substrates for the whole cell biocatalyst in further experiments. If successful, this would be a significant step forward in the chemoenzymatic synthesis of anti cancer agents.

Experimental Section

Bacterial strains, plasmids and culture conditions

Escherichia coli strains UT5600(DE3) [F^-, *ara*-14, *leu*B6, *sec*A6, *lac*Y1, *pro*C14, *tsx*-67, Δ(*omp*T-fepC)266, *ent*A403, *trp*E38, *rfb*D1, *rps*L109-(Str^r), *xyl*-5, *mtl*-1, *thi*-1, λ(DE3)], and BL21(DE3) [B, F^-, *dcm*, *omp*T, *lon*, *hsdS*(rB^- mB^-), *gal*, λ(DE3)] were used for the expression of autotransporter fusion proteins. *E.coli* TOP10 (F^- *mcr*A Δ(*mrrhsd*RMS-*mcr*BC) ϕ80 L*ac*ZDM15 Δ*lac*X74 *deo*R *rec*A1 *ara*D139 Δ(*ara-leu*) 7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G) and the vector pCR4-TOPO which were used for subcloning of PCR products were obtained from Invitrogen. Plasmid pET-Adx04, which encodes the AIDA-I autotransporter, and plasmid pHis8-PT2 (pIU18), which encodes dimethylallyltryptophansynthase FgaPT2, have been described elsewhere.[16,22] Bacteria were routinely grown at 37 °C in Lysogeny broth (LB) containing ampicillin (100 mg L^{-1}). For FgaPT2, expression studies and enzyme assays cells were cultured in the presence of ethylendiamintetraacetate (EDTA, 10 μM) and 2-mercaptoethanol (10 mM).

Recombinant DNA techniques

For construction of plasmid pEK004, which contains the gene encoding the FgaPT2-autotransporter fusion protein, the *fgaPT2* gene was amplified by PCR. Plasmid pHis8-PT2 (pIU18) was used as template for primers EK003 (5′-CTGCTCGAGATGAAGGCAGCCAATGCCTC−3′) and EK004 (5′-GGGGTACCGTGGAGACCGGAATAATATACC−3′). The PCR product was inserted into vector pCR4-TOPO and recleaved with XhoI and KpnI. The restriction fragment was ligated to pET-Adx04, restricted with the same enzymes. This yields an in frame fusion of *fgaPT2* with the autotransporter domains under the control of a T7/lac promoter (Figure 3) as described by Petermann et al.[35] Preparation of plasmid DNA, ligation, restriction digestion, transformation procedures and DNA electrophoresis were performed according to standard protocols[36].

Outer membrane preparation

E. coli cells were grown overnight and 1 mL of the culture was used to inoculate LB medium (40 mL). Cells were cultured at 37 °C with vigorous shaking (200 rpm) for about 2 h until an OD_{578} of 0.5 was reached. The culture was separated into two aliquots and protein expression was induced by adding IPTG at a final concentration of 1 mM to one of the aliquots. Cultures then were incubated at 30 °C and shaking (200 rpm) for one hour. Induction was stopped by incubating the cells on ice for 15 min. After harvesting and washing of the cells with Tris-HCl (0.2 M, pH 8), differential cell fractionation was performed according to the method of Hantke[37] as modified by Schultheiss et al.[37–38]

The outer membrane proteins were washed, resuspended in water and prepared for SDS-PAGE.

For whole cell protease treatment, *E. coli* cells were harvested, washed and resuspended in 1 mL Tris-HCl (0.2 M, pH 8). Proteinase K was added to a final concentration of 0.2 mg mL^{-1} and cells were incubated for 1 hour at 37 °C. Digestion was stopped by washing the cells twice with Tris-HCl (0.2 M, pH 8) containing 10% fetal calf serum (FCS) and outer membrane proteins were prepared as described above.

SDS-PAGE and Western Blot Analysis

Outer membrane isolates were diluted (1:1.5) with sample buffer (100 mM Tris/HCl (pH 6.8) containing 4% SDS, 0.2% bromophenol blue, 200 mM dithiothreitol and 20% glycerol), boiled for 5 min and analyzed on 10% SDS-PAGE. Proteins were stained with Coomassie brilliant blue (R250).

For Western Blot analysis, gels were electroblotted on polyvinylidenefluoride (PVDF) membranes. Blotted membranes were blocked in TBS with 3% bovine serum albumin (BSA). For immune detection, membranes were incubated with the primary anti-FgaPT2 polyclonal antibody serum diluted 1:2000 in TBS with 3% BSA for 3 h. Prior to addition of the secondary antibody, immunoblots were rinsed three times in TBS with 0.1% Tween 20. The secondary antibody was added and the blots were incubated for 2 h at room temperature.

Antigen-antibody conjugates were visualized by treatment with horseradish peroxidase linked goat anti-rabbit IgG diluted 1:5000 in TBS with 3% BSA. A color reaction was achieved by adding a solution consisting of 4-chloro-1-naphthol (3 mg mL^{-1}) in methanol (5 mL), TBS (25 mL), and H_2O_2 (30%, 15 μL).

Chemicals for prenyltransferase assay

Trisammonium salt of DMAPP was synthesized in analogy to trisammonium geranyl diphosphate synthesis reported by Woodside et al..[39] L-β-homotryptophan was purchased from Fluka (Seelze, Germany) and indole-3-propionic acid from Aldrich (Seelze, Germany).

Conditions for enzymatic reactions

For determination of enzymatic activity of surface displayed FgaPT2, reaction products were measured by using HPLC. For this purpose cells were grown over night and then used 1:100 to inoculate a fresh culture. At an OD_{578} of 0.5, protein expression was induced by adding 1 mM of IPTG and cells were incubated for 4 h at 30 °C and 200 rpm. After induction cells were washed twice with Tris-HCl 50 mM pH 7.5, resuspended in the same buffer to an OD_{578} of 50 and stored on ice. The assay with His_8-FgaPT2 was performed as described previously.[16] The standard assay for determination of activity of the displayed FgaPT2 (100 μL) was performed in Tris-HCl 50 mM pH 7.5 and contained 1 mM tryptophan derivative, 1–10 mM DMAPP and 80 μL cell suspension ($OD_{578}=40$). The reaction mixtures were incubated for 24 h at 20–35 °C. After removal of the cells by centrifugation for 10 min at 14000×g and −4 °C, the supernatant was transferred into a new tube and protein was precipitated by 1 volume methanol. After centrifugation, the supernatant was analyzed by HPLC and LC-MS as described below. Two independent assays were performed for quantification.

HPLC analysis

Reaction mixtures were analyzed by using an HP HPLC Series 1090 by using a Multospher 120 RP18 (5 μm, 250×4 mm) at a flow rate of 1 mL min^{-1}. Water (solvent A) and acetonitrile (solvent B), each containing 0.5% trifluoroacetic acid, were used as solvents. A gradient was run from 15% to 70% B in 13 min. After washing with 100% solvent B for 5 min, the column was equilibrated with 15% solvent A for 5 min. The substances were detected by using a photo diode array detector and illustrated for absorption at $\lambda=$ 296 nm.

Spectroscopic analysis

The assays were analyzed by using positive and negative electrospray ionization (ESI) mass spectrometry with a ThermoFinnigan TSQ Quantum. The mass spectrometer was coupled with an Agilent HPLC series 1100 equipped with a RP18-column (2×250 mm, 5 μm). For separation, the column was run with 10% solvent B (methanol) in solvent A (water, each containing 0.1% HCOOH) for 5 min, followed by a gradient from 10% to 100% B over 30 min. After washing with 100% B, the column was equilibrated with 10% B for 10 min. The flow rate was at 0.2 mL·min^{-1}.

Acknowledgements

We thank Alexander Grundmann for the synthesis of DMAPP. This work was funded by grants from the Deutsche Forschungsgemeinschaft to Shu-Ming Li and Joachim Jose.

Keywords: Autodisplay · biocatalysis · chemoenzymatic synthesis · transferases · enzymes

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Received: December 3, 2010
Published online on May 30, 2011

