Archives of Microbiology (2020) 202:1419–1424
https://doi.org/10.1007/s00203-020-01854-7

ORIGINAL PAPER

Reinvestigation of the substrate specificity of a reverse prenyltransferase NotF from *Aspergillus* sp. MF297-2

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Received: 13 November 2019 / Revised: 15 February 2020 / Accepted: 3 March 2020 / Published online: 17 March 2020

Abstract

NotF from *Aspergillus* sp. MF297-2 and BrePT from *Aspergillus versicolor* catalyze a reverse C2-prenylation of brevianamide F in the biosynthetic pathway of brevianamides and notoamides. NotF was reported to use only brevianamide F as substrate while BrePT demonstrated broad substrate promiscuity. With high identity at amino acid level, it is interesting to reinvestigate the catalytic activities of these two prenyltransferases in vitro toward 14 cyclodipeptides. Product identification of the in vitro assays by MS proved that NotF and BrePT share similar catalytic ability and substrate promiscuity.

Keywords Prenyltransferase · DMATS · NotF · BrePT · Enzyme catalysis · Substrate promiscuity

Introduction

Prenyltransferases are a large family of enzymes found in all domains of life and are involved in the biosynthesis of primary and secondary metabolites, such as cis-/trans-prenyltransferases for isoprenoids (Fig. 1a) (Heide 2009; Li et al. 2015; Winkelblech et al. 2015). They catalyze the transfer reactions of prenyl moieties from different prenyl donors, like dimethylallyl, geranyl, or farnesyl, to various aliphatic or aromatic acceptors including proteins and nucleic acids (Fig. 1b) (Heide 2009; Li 2009; Palsuledesai and Distefano 2015; Yazaki et al. 2009). Enzymes, which use aromatic substrates as prenyl acceptors, are known as aromatic prenyltransferases and found in plant, bacteria and fungi (Fig. 1c) (Heide 2009; Li 2009; Yazaki et al. 2009). One subgroup of these prenyltransferases from ascomycetes shares significant sequence similarities with dimethylallyltryptophan synthase (DMATS) in the biosynthesis of ergot alkaloids from *Claviceps purpurea* (Tudzynski et al. 1999), and are classified as prenyltransferases of the DMATS superfamily. They catalyze usually regioselective and stereoselective prenylations of a series of aromatic substances including tryptophan, tryptophan-containing peptides, and other indole derivatives as well as tyrosine or even nitrogen-free substrates (Winkelblech et al. 2015). Until now, more than 50 of such soluble enzymes are identified and characterized biochemically (Winkelblech et al. 2015; Wohlgemuth et al. 2017).

Tryptophan-containing cyclic dipeptide prenyltransferases catalyze regiospecific prenylations at different positions of the indole ring (Fig. 1c) (Li et al. 2015; Winkelblech et al. 2015). FtmPT1 from *Aspergillus fumigatus* (*A. fumigatus*) was identified as the first cyclic dipeptide prenyltransferase and catalyzes a regular C2-prenylation of brevanamide F (*cyclo*-L-Trp-L-Pro) in the biosynthesis of fumitremorgins (Grundmann and Li 2005; Li 2011). NotF from an *Aspergillus* sp., BrePT from *A. versicolor* and CdpC2PT from *Neosartorya fischeri* were latter identified (Ding et al. 2010; Mundt and Li 2013; Yin et al. 2013), which catalyze reverse C2-prenylation of tryptophan-containing cyclic dipeptides.

Communicated by Olaf Kniemeyer.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00203-020-01854-7) contains supplementary material, which is available to authorized users.

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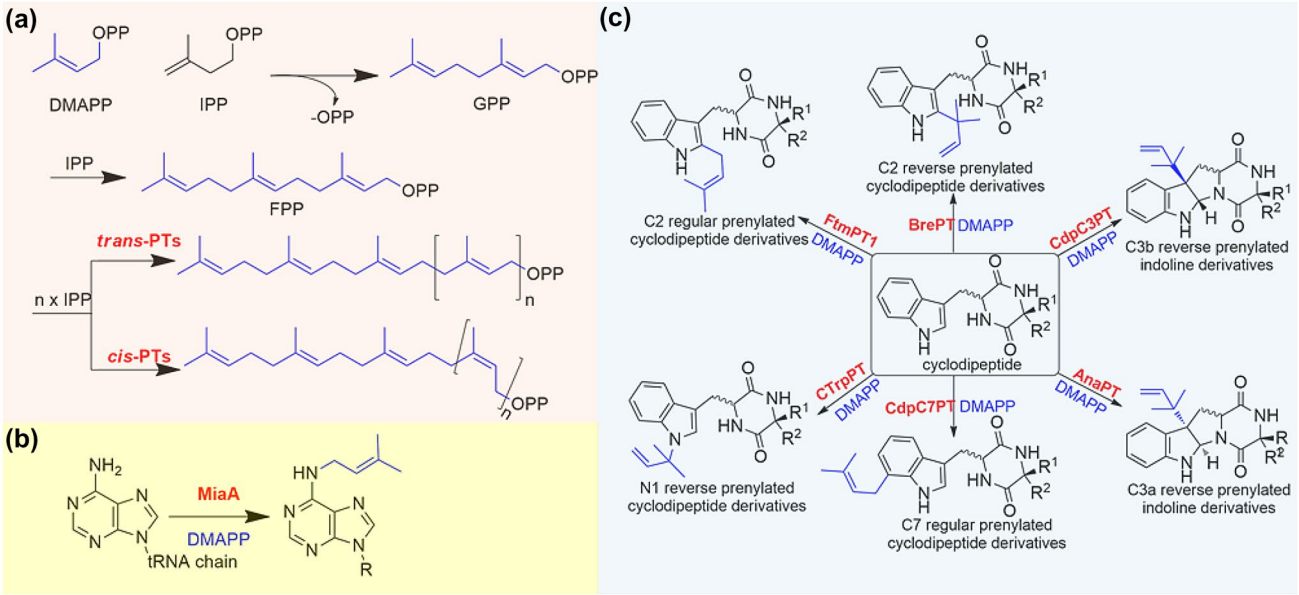

Fig. 1 Examples of prenyltransferases **a** *Cis-/trans*-PT for the carbon skeleton biosynthesis of isoprenoids; **b** t-RNA prenyltransferase MiaA from *E. coli*; **c** Examples of DMATS enzymes catalyzing the prenylations of cyclodipeptides

NotF from *Aspergillus* sp. MF297-2 is involved in the biosynthesis of notoamides and known as only used brevianamide F as the substrate (Ding et al. 2010). In contrast, the homologous protein BrePT from *A. versicolor* is reported to be able to prenylate 14 tryptophan-containing cyclic dipeptides (Yin et al. 2013). The high sequence identity of 83% at the amino acid level (Supplementary Fig. S1) between NotF and BrePT raised our interest to compare their substrate specificity.

Materials and methods

Chemicals

Dimethylallyl diphosphate (DMAPP) was prepared according to the method for geranyl diphosphate (Woodside et al. 1988). The four *cyclo*-Trp-Pro isomers were synthesized from tryptophan methyl ester and N-Boc protected proline according to the method published previously (Caballero et al. 2003). 13a was synthesized from N-Boc-L-Trp-OH and H–L-His-OMe·HCl according to the publications (Bivin et al. 1993). The four stereoisomers of *cyclo*-Trp-Ala, 9a, 10a, 11a, 12a and 14a were prepared in a similar way (Bivin et al. 1993). N-Chlorosuccinimide (NCS), geraniol, disodium dihydrogen pyrophosphate, amino acids, dichloromethane, dimethyl sulfide, sodium chloride, and acetonitrile of the highest available purity were purchased from TCI, Sigma-Aldrich, and Bachem.

Computer-assisted sequence analysis

Sequence identities were obtained by alignments of amino acid sequences using the "BLAST 2 sequences" (www.ncbi.nlm.nih.gov) (Supplementary Fig. S1).

Bacterial strains, plasmids and culture conditions

The vector pET-28a was obtained from Novagen (Beijing, China). *Escherichia coli* DH5α (Invitrogen) and BL21(DE3) (Qiagen, Hilden, Germany) were used as cloning and expression hosts, respectively. They were grown in liquid Luria–Bertani (LB) medium or Terrific broth (TB) medium and on solid LB medium with 1.5% (w/v) agar at 30 °C or 37 °C. 50 $\mu g \cdot ml^{-1}$ of kanamycin were used for selection of recombinant *E. coli* cells.

DNA propagation in *E. coli*., PCR amplification and gene cloning

pUC-SP-*NotF* (Sangon, Shanghai, China) was used as template, and NotF-f (5′- ATTGGATCCATGACCGCG CCGGAACTG -3′) and NotF-r (5′- ATTAAGCTTTTA ATCTTCTTCCCACAG-3′) as primers. The underlined sequences represent the restriction sites *BamHI* and *HindIII* for cloning in pET-28a vector. The PCR amplifications were carried out at 55 °C as annealing temperature on an iCycler from BioRad with Expand High Fidelity PCR Kit (Roche Diagnostic, Mannheim, Germany). The fusion PCR

product was digestion with BamHI and HindIII, cloned into the corresponding sites of pET-28a vector, and subsequently sequenced to verify the gene sequence (Seqlab Sequence Laboratories, Göttingen, Germany).

Overproduction and purification of His_6-BrePT and His_6-NotF

His_6-NotF was produced in *E. coli* BL21(DE3) cells. The plasmid pET-28a-*NotF* were transformed into *E. coli* BL21(DE3) cells, which were cultivated in 2000-ml Erlenmeyer flasks containing 1000 ml liquid LB medium with kanamycin (50 μg·ml^{-1}) at 37 °C to A_{600} of 0.6 and induced with 0.5 mM isopropyl thiogalactoside (IPTG) for overnight at 37 °C. For overproduction of His_6-BrePT, *E. coli* BL21(DE3)pLysS cells harboring the plasmid pSY1 were cultivated in 2000-ml Erlenmeyer flasks containing 1000 ml of liquid LB medium, supplemented with kanamycin (50 μg·ml^{-1}) and grown at 37 °C to an absorption of 0.7 at 600 nm. For induction, IPTG was added to a final concentration of 0.1 mM, and the cells were cultivated for a further 5 h at 20 °C before harvest (Yin et al. 2013).

The bacterial cultures were centrifuged, and the pellets were resuspended in lysis buffer (10 mM imidazole, 50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0) at 2–5 ml/g wet weight. After addition of 1 mg·ml^{-1} lysozyme and incubation on ice for 30 min, the cells were sonicated six times for 10 s each at 200 watts. To separate the cellular debris from the soluble proteins, the lysate was centrifuged at 13,000×*g* for 30 min at 4 °C. One-step purification of the recombinant His_6-tagged fusion protein by affinity chromatography with nickelnitrilotriacetic acid-agarose resin (Qiagen) was carried out according to the manufacturer's instructions. The protein was eluted with 250 mM imidazole in 50 mM NaH_2PO_4, 300 mM NaCl (pH 8.0). To change the buffer, the protein fraction was passed through a PD-10 column (GE Healthcare), which had been previously equilibrated with 50 mM Tris–HCl, 15% (v/v) of glycerol (pH 7.5). BrePT or NotF was eluted with the same buffer and frozen at − 80 °C for enzyme assays (Yu and Li 2012).

Assays for determination of NotF and BrePT activity

For determination of the NotF activity, reaction mixtures (50 μl) contained 50 mM Tris–HCl (pH 7.5, 37 °C), 10 mM $CaCl_2$, 2 mM cyclic dipeptide, 2 mM DMAPP, glycerol (0.5–5%, v/v), DMSO (2.5%, v/v) and 0.85 μM of purified recombinant NotF or BrePT and were incubated at 37 °C for 4 h. Up to 5% DMSO in the reaction mixtures had no influence on the enzyme activity (Yu, Liu et al. 2012). The reactions were terminated by addition of 50 μl methanol. After centrifugation at 13,000×*g* for 20 min to remove protein, the enzyme products were analyzed by HPLC under the following conditions.

HPLC conditions for analysis the enzyme products

An Agilent HPLC series 1200 was used for analysis the enzyme products of NotF using a Multospher 120 RP-18 column (250×4 nm, 5 μm, CS Chromatographie Service, Langerwehe, Germany) at 1 ml min^{-1} flow rate. Water (solvent A) and acetonitrile (solvent B) were used as solvents with a linear gradient of 10–90% (v/v) solvent B in 20 min. The column was then washed with 90% solvent B for 5 min, and equilibrated with 10% (v/v) solvent B for 5 min. Detection was carried out on a photodiode array detector and illustrated at 254 nm.

LC–MS for structure elucidation

The enzyme products were also analyzed by LC–MS on an Agilent HPLC 1260 series system with a Bruker micro TOF-Q III mass spectrometer (Bruker, Bremen, Germany) with an ESI source. Separation was performed on an Agilenet Eclipse XDB-C18 column (150×4.6 mm, 5 μm) with a linear gradient of 5–100% (v/v) acetonitrile (solvent B) in water (solvent A), both contain 0.5% (v/v) trifluoroacetic acid, in 40 min using 0.5 ml min^{-1} flow rate. The column was then washed with 100% (v/v) solvent B for 5 min and quilibrated with 5% (v/v) solvent B for 5 min.

Results

NotF overproduction and purification

Notoamide gene cluster was analyzed from a marine-derived *Aspergillus sp*. MF297-2 using genome mining via Roche 454FLX technology, published in 2010 (Ding et al. 2010). NotF was a part of this cluster, identified as a reverse prenyltransferase, involved in the C2-prenylation of brevianamide F, resulting in the formation of deoxybrevianamide E. In addition, NotF was reported to be highly specific toward its natural substrate and only accepted *cyclo*-L-Trp-L-Pro for prenylation (Ding et al. 2010).

NotF is encoded by a nucleotide sequence of 1431 bp (GenBank acccession number GU564535). The coding sequence was synthesis by Sangon (Shanghai, China) and cloned into pET-28a vector for protein overproduction. After optimization of induction conditions, *E. coli* BL21(DE3) harboring the expression plasmid was inoculated in TB medium and induced with 0.5 mM IPTG at 37 °C overnight. Recombinant protein was purified by affinity chromatography on Ni–NTA agarose and analyzed on SDS-PAGE (Supplementary Fig. S2). The protein with the desired molecular

weight at 53 kDa was obtained (Ding et al. 2010). No difference was observed between the activities of the His-tagged and non-tagged protein (Haagen, Unsold et al. 2007). Therefore, the purified his-tagged protein was used for further enzyme assays.

Acceptance of 14 tryptophan-containing cyclic dipeptides by NotF in the presence of DMAPP

In the previous study, NotF has been tested with 5 substrates including its natural substrate brevianamide F, L-Trp, *cyclo*-L-Trp-L-Trp, *cyclo*-L-Phe-L-Pro and *cyclo*-L-Trp-L-Tyr. With all the unnatural substrates, no catalytic activity was detected by LC–MS (Ding et al. 2010). To better compare their activities, we incubated NotF and BrePT parallelly with 14 tryptophan-containing cyclic dipeptides. HPLC analysis of the incubation mixtures revealed that NotF was able to accept all of four stereoisomers of *cyclo*-L-Trp-L-Pro as well as *cyclo*-L-Trp-L-Ala (Fig. 2 and Supplementary Fig. S3-S10). The best accepted substrates by NotF are its natural substrate *cyclo*-L-Trp-L-Pro (1a) and one stereoisomer *cyclo*-L-Trp-D-Pro (2a) with conversion yields of 66% and 61%, respectively (Fig. 2, Supplementary Fig. S3A and S4A). While the other two isomers *cyclo*-D-Trp-L-Pro (3a) and *cyclo*-D-Trp-D-Pro (4a) were converted by NotF with conversion yields of 18% and 6%, respectively (Fig. 2, Supplementary Fig. S5A and S6A). It seems that the stereochemistry of tryptophanyl moiety in the substrate is important for the acceptance by NotF. Interestingly, different results were observed for the four isomers of *cyclo*-Trp-Ala. For *cyclo*-L-Trp-L-Ala (5a) and *cyclo*-D-Trp-L-Ala (7a), NotF showed higher conversion yields of 11% and 17%, respectively (Fig. 2, Supplementary Fig. S7A and S9A). In contrast, the conversion yields of *cyclo*-L-Trp-D-Ala (6a) and *cyclo*-D-Trp-D-Ala (8a) are only 5% and 1%, respectively (Fig. 2, Supplementary Fig. S8A and S10A).

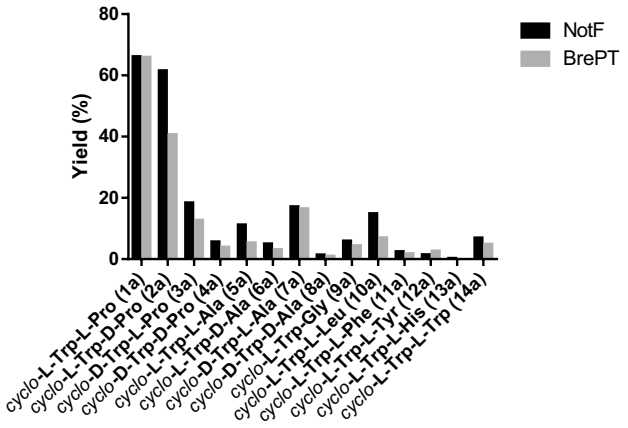

Fig. 2 Product yields of NotF and BrePT toward cyclodipeptides

It seems that the stereochemistry of alanine moiety here is more important than that of tryptophan, which indicates that *cyclo*-Trp-Pro and *cyclo*-Trp-Ala might interact with NotF differently. In comparison to 5a, *cyclo*-L-Trp-Gly (9a) with a similar structure showed lower yield of 6%, which is similar to that of *cyclo*-L-Trp-L-Trp (14a). *Cyclo*-L-Trp-L-Leu (10a) was accepted well by NotF with conversion yield of 15%. *Cyclo*-D-Trp-D-Ala (8a), *cyclo*-L-Trp-L-Phe (11a) *cyclo*-L-Trp-L-Tyr (12a), *cyclo*-L-Trp-L-His (13a) and *cyclo*-L-Trp-L-Trp (14a) were also accepted by NotF, but with yields less than 3%.

Reverse C2-prenylation of tryptophan-containing cyclic dipeptides

For structural elucidation, the reaction mixtures of NotF were subjected to LC–MS analysis. HRMS data (Supplementary Table S1) revealed the presence of molecular masses, which are 68 Da larger than those of their respective substrates, providing evidence for monoprenylation in all the products.

In the tandem secondary mass spectra (MS^2), five main peaks were observed and marked as ion A ($[M+H]^+$), ion B ($[M+H\text{-}C_5H_9]^+$), fragment C, fragment D, and fragment E (Fig. 3 and Supplementary Table S1). The main peaks of products (1b-13b) are ion B, indicating the structure of products lacking the isopentenyl group. Differing from 14b, the major peak is its quasi-molecular ion (ion A). Fragments C and D were observed in all the reaction mixtures indicating the presence of the 2-prenylated 3-methylindole residue and the 3-methylindole residue, respectively (Fig. 3). For 1b and 4b, m/z 170.0608 was observed and marked as fragment E, indicating the presence of derivative of fragment B.

The LC-ESI-MS^2 data of the products of BrePT are listed in Supplementary Table S2. The enzyme products of NotF and BrePT share the same molecular weight, and the results of the tandem secondary mass spectra are also almost identical (Supplementary Fig. S3-S16). Based on these results, it is plausible that their products are the same C2-prenylated derivatives.

Discussion

Prenylated compounds are a large group of secondary metabolites and widely distributed in nature (Heide 2009). In comparison to their nonprenylated precursors, prenylated compounds exhibit more potent biological and pharmacological activities in general. Prenyltransferases of the DMATS superfamily are capable of prenylating a wide range of substrates. Previously study shows NotF had a high specificity for brevianamide F and 12a and 14a were not accepted (1). In this study, NotF from *Aspergillus*

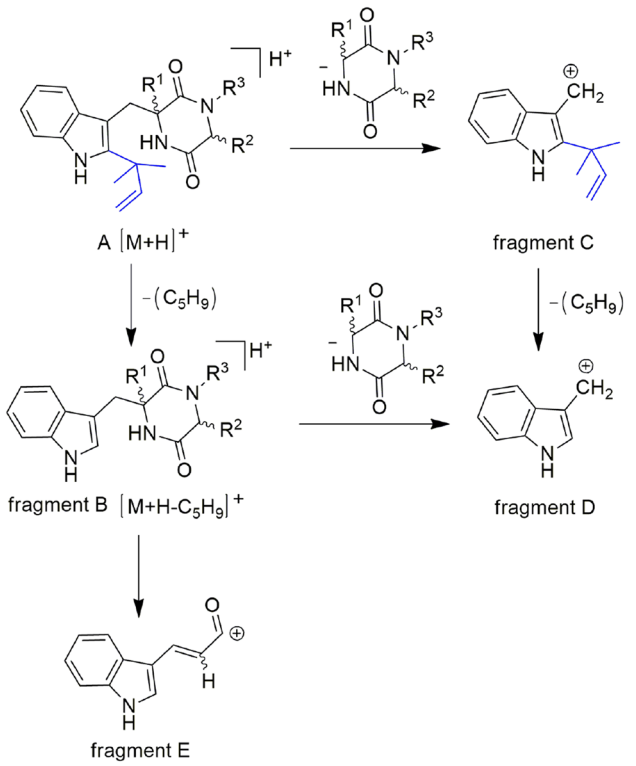

Fig. 3 Key ions and fragments of the products

sp. MF297-2 was overproduced and purified as described by Ding et al. (2010). The substrate specificity of NotF was tested with 14 tryptophan-containing cyclic dipeptides under the same condition as BrePT. The results showed that NotF consumed all the substrates with varied catalytic activities, but similar to BrePT (Fig. 2). Both enzymes accepted 1a as the best substrate and 2a as the second with conversion yields of more than 40%. For other tryptophan containing cyclic dipeptides with an aromatic (11a, 12a, 14a) or heterocyclic amino acid (13a) as the second amino acid moiety were poorly accepted by both NotF and BrePT. In addition, amino acids with D-configurations are usually poor substrates, with an exception for substrates 5a and 7a, indicating that they might be placed in the active pocket differently.

In conclusion, reinvestigation the substrate promiscuity of NotF revealed that it is capable of prenylating a wide range of cyclic dipeptides as well and could also be a promising biocatalyst in the future.

Acknowledgements This work was supported by the National Natural Science Foundation of China (81803409 and 21838001) and the Fundamental Research Funds for the Central Universities (grant numbers buctrc201810, XK1802-8 and PT1909).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standard Research does not involve Human Participants and/or Animals.

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