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Tryptophan Aminopeptidase Activity of Several Indole Prenyltransferases from *Aspergillus fumigatus*

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SUMMARY

Recently, five indole prenyltransferases from *Aspergillus fumigatus* have been proven biochemically to be responsible for prenylations of diverse substrates. In this study, we show peptidase activities of 7-DMATS, FgaPT1, CdpNPT, and FtmPT1, with preference for linear peptides containing a tryptophanyl moiety at the N terminus. Testing of 31 peptides revealed that these enzymes shared similar substrate specificity and accepted H-L-Trp-L-Ala-OH and H-L-Trp-Gly-OH as best substrates for aminopeptidase activity. By using H-L-Trp-Gly-OH as substrate, K_m values at 350, 380, 300, and 420 μM and enzymatic rate constants k_{cat}/K_m at 0.51, 0.24, 0.53, and 0.14 $mM^{-1}s^{-1}$ were determined for 7-DMATS, FgaPT1, CdpNPT, and FtmPT1, respectively. In contrast to prenyltransferase activities, the aminopeptidase activities were strongly or completely inhibited by EDTA. Mn^{2+} increased the aminopeptidase activities of FtmPT1 and CdpNPT up to 4- and 6-fold, respectively. To the best of our knowledge, this is the first report on the catalytic promiscuity of prenyltransferases.

INTRODUCTION

From the genome sequence of *Aspergillus fumigatus* AF293, seven putative prenyltransferase genes could be identified by bioinformatic approach that share sequence similarity to the dimethylallyltryptophan synthase from *Claviceps purpurea* (Nierman et al., 2005). They belong probably to five different biosynthetic gene clusters of secondary metabolites (Nierman et al., 2005). Recently, we have characterized five of these genes from four biosynthetic gene clusters by gene cloning, expression, and biochemical investigation (Grundmann and Li, 2005; Kremer et al., 2007; Ruan et al., 2008; Unsöld and Li, 2005, 2006; Yin et al., 2007). Our results proved that these enzymes catalyze different prenylations at various positions of the indole rings of diverse secondary metabolites (Figure 1); e.g., FgaPT2 and FgaPT1 are involved in the biosynthesis of fumigaclavine C and catalyze the prenylation of L-tryptophan at position C4 and the prenylation of fumigaclavine A at position C2 of the indole rings, respectively (Figures 1A and 1E; Unsöld and Li, 2005, 2006). Therefore, FgaPT2 functions as a 4-dimethylallyltryptophan synthase. A second dimethylallyltryptophan synthase, 7-DMATS, catalyzes the prenylation of L-tryptophan at position C7 of the indole ring (Figure 1B; Kremer et al., 2007). From a gene cluster of fumitremorgins, FtmPT1 was proven to catalyze the prenylation of cyclo-L-Trp-L-Pro (brevianamide F) at position C2 of the indole ring (Figure 1C; Grundmann and Li, 2005). The very recently identified prenyltransferase, CdpNPT, was found to catalyze the reverse prenylation of tryptophan-containing cyclic dipeptides at position N1 of the indole rings (Figure 1D; Ruan et al., 2008). During the investigation on 7-DMATS, it was found out that this enzyme accepted also tryptophan-containing cyclic dipeptides and linear dipeptides such as H-L-Trp-Gly-OH as substrates (Kremer et al., 2007). Surprisingly, two product peaks were detected in the incubation mixture of H-L-Trp-Gly-OH, dimethylallyl diphosphate (DMAPP) and the recombinant His_6-7-DMATS (Figure 2A), but not in the control incubation with heat-inactivated protein (Figure 2B). These compounds were identified as H-L-7-DMAT-Gly and 7-dimethylallyltryptophan (7-DMAT) (Figure 1F) after preparative isolation and structural elucidation by NMR and MS analysis (unpublished data). The presence of 7-DMAT in the reaction mixture indicates a hydrolytic process after or before the prenylation reaction during the incubation, i.e., hydrolysis of the expected prenylated product H-L-7-DMAT-Gly to 7-DMAT or hydrolysis of the substrate H-L-Trp-Gly-OH to L-Trp, which was subsequently prenylated to 7-DMAT. In both cases, peptidase activity of the enzyme should be involved. To our knowledge, no peptidase activity of a prenyltransferase is described in the literature. In this study, we report on the aminopeptidase activities of the indole prenyltransferases from *Aspergillus fumigatus*.

RESULTS

Peptidase Activities of the Prenyltransferases

As shown in Figure 2, the substrate H-L-Trp-Gly-OH was chemically stable under the condition for enzymatic reactions and no additional peak was detected in the incubation mixtures with heat-denaturated recombinant proteins. HPLC analysis of the incubation mixture of H-L-Trp-Gly-OH and His_6-7-DMATS in the absence of DMAPP showed one product, which was absent in the control assay with heat-inactivated His_6-7-DMATS.

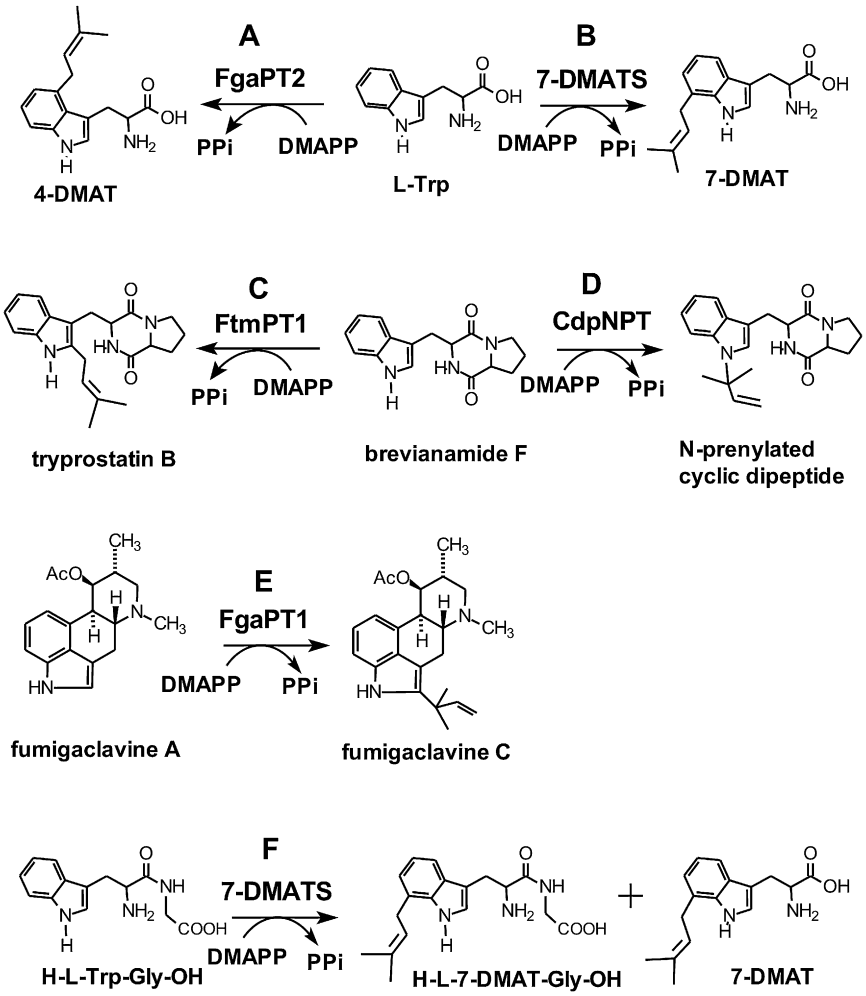

Figure 1. Prenyltransfer Reactions Catalyzed by Different Prenyltransferases from *Aspergillus fumigatus*

(A and B) Conversion of L-Trp to 4-dimethylallyltryptophan by FgaPT2 (A) and to 7-dimethylallyltryptophan by 7-DMATS (B).

(C and D) Conversion of brevianamide F by FtmPT1 to tryprostatin B (C) and to N-prenylated cyclic dipeptide by CdpNPT (D).

(E) Conversion of fumigaclavine A to C by FgaPT1.

(F) Conversion of H-L-Trp-Gly-OH to H-L-7-DMAT-Gly-OH and 7-dimethylallyltryptophan by 7-DMATS.

Incubation with 0.6 μM protein for 16 hr resulted in complete conversion of H-L-Trp-Gly-OH to the enzymatic product (Figure 2C). The enzymatic product could be identified as L-Trp by cochromatography on HPLC and by LC-MS analysis. Positive ESI-LC-MS analysis of the reaction mixture indicated the presence of $[M+1]^+$ for tryptophan at m/z 205 and $[M+1]^+$ for glycine at m/z 76. This result proved that the indole prenyltransferase 7-DMATS showed indeed a peptidase activity and hydrolysis of the dipeptide H-L-Trp-Gly-OH to the amino acids tryptophan and glycine has taken place during the incubation. To prove whether other purified and identified indole prenyltransferases also shared this property, peptidase activities of FgaPT1, CdpNPT, FtmPT1, and FgaPT2 were tested by using H-L-Trp-Gly-OH as substrate in the presence of Ca^{2+}, but in the absence of DMAPP. As shown in Figure 2, product formation was clearly detected with recombinant FgaPT1, which is absent in the negative control (Figures 2E and 2F). A conversion rate of 63% could be achieved after incubation for 16 hr. In the presence of 5 mM of Ca^{2+}, CdpNPT and FtmPT1 showed lower activities than 7-DMATS and FgaPT1, with conversion rates of 17.7 and 10.3%, respectively (Figures 2G and 2K). On the other hand, another dimethylallyltryptophan synthase FgaPT2 showed no detectable peptidase activity under this condition (Figures 2O and 2P). These data proved that FgaPT1, CdpNPT, and FTmPT1 are also active as peptidases.

Influence of Metal Ions and ATP on the Peptidase Activities

Our previous results on the prenyltransfer activities of the prenyltransferases showed that the enzymatic activities were not inhibited by addition of the metal chelating agent EDTA (Grundmann and Li, 2005; Kremer et al., 2007; Unsöld and Li, 2005; Unsöld and Li, 2006; Yin et al., 2007), indicating that metal ions were not essential for the prenyltransfer reactions, although Ca^{2+} ions enhance the reactions of some enzymes in this group. Investigation of the peptidase activity of 7-DMATS by using H-L-Trp-Gly-OH as substrate showed that no significant change of the enzyme activity was observed by addition of divalent metal ions such as Ca^{2+}or Mg^{2+} to a final concentration of 5 (Table 1) or 10 mM (data not shown). It seems that metal ions were also not essential for the peptidase activity. In the incubation mixture with EDTA, however, the peptidase activity of 7-DMATS was reduced to 11.6% of that of the incubation mixture without additives. This indicated strongly that divalent metal ions were important for the enzymatic activity. Similar results were also obtained for the peptidase activity of FgaPT1, FtmPT1 and CdpNPT: no or only slight increasing (150% or less) of enzyme activities was observed by addition of metal ions such as Ca^{2+} and Mg^{2+}. However, more than 50% inhibition of the peptidase activity of FgaPT1 was detected after addition of EDTA to the incubation mixture. Peptidase activities of CdpNPT and FtmPT1 were completely inhibited by 5 mM EDTA (Table 1). To explain this contra verse phenomenon, dependence of peptidase activities of 7-DMATS, FgaPT1, FtmPT1, and CdpNPT on additional metal ions was investigated. The enzymatic activities of 7-DMATS and FgaPT1 were reduced in the assays with Mn^{2+} to 84 and 70% of those of incubations without additives, respectively. In contrast, the peptidase activities of FtmPT1 and CdpNPT have been significantly increased in the presence of Mn^{2+} up to four- and six-fold, respectively. These data proved that Mn^{2+} is essential for the peptidase activities

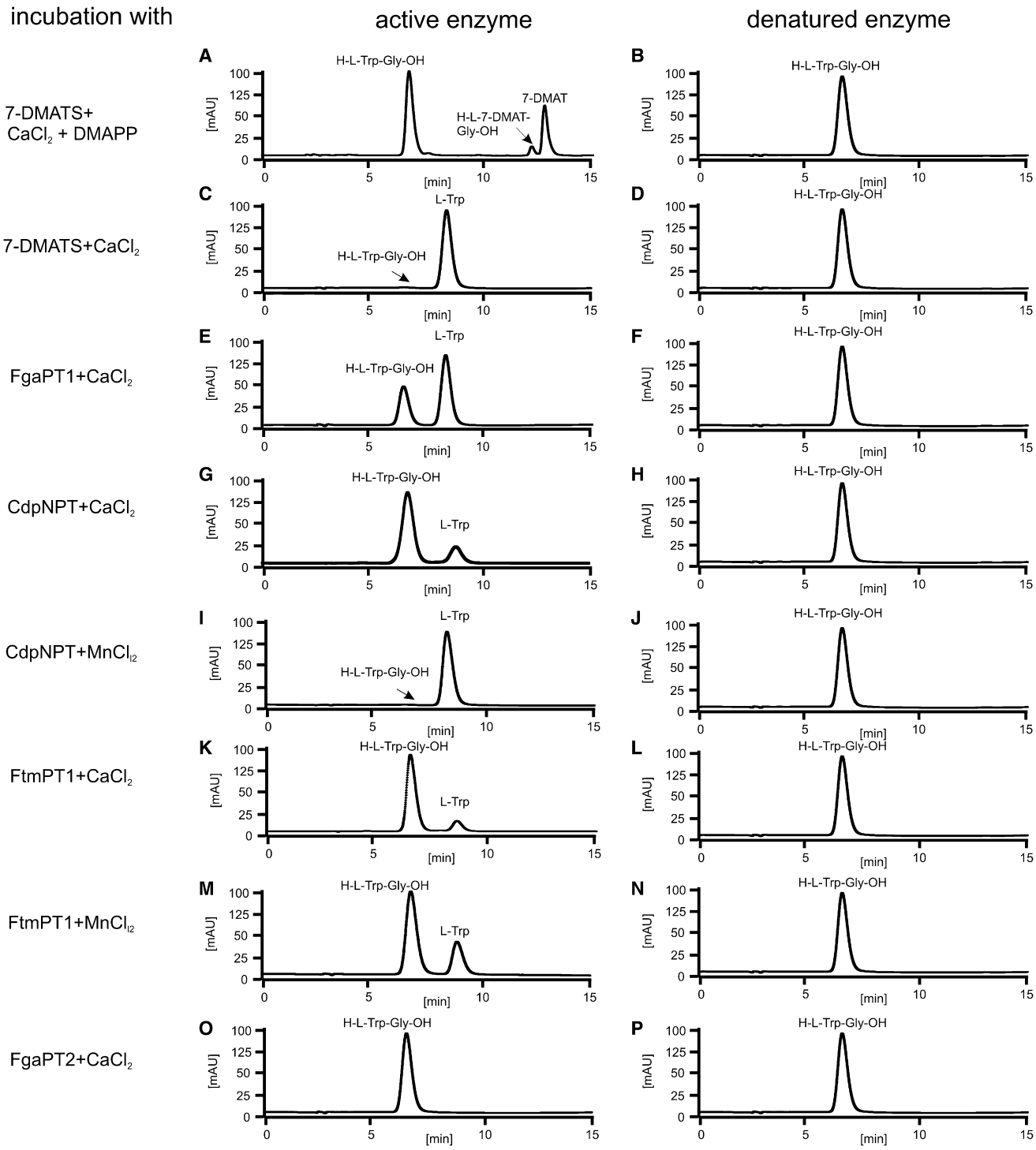

Figure 2. Hydrolysis of H-L-Trp-Gly-OH by Different Prenyltransferases under Different Conditions

(A–P) The incubation mixtures contained 2 mM substrate, 5 mM metal ions, and 0.6 μM 7-DMATS and FtmPT1 or 0.3 μM CdpNPT and FgaPT1 and were incubated at 37°C for 16 hr. Detection was carried out at 277 nm.

of these two enzymes. By using H-L-Trp-Gly-OH as substrate, FtmPT1 showed a maximum of peptidase activity in the presence of Mn^{2+} at a final concentration of 2.5 to 30 mM (data not shown). Incubation of CdpNPT with H-L-Trp-Gly-OH in the presence of Mn^{2+} at a final concentration of 5 mM for 16 hr resulted in complete conversion of the substrate to L-Trp (Figure 2I). A conversion rate of 28% could be achieved with FtmPT1 in the presence of Mn^{2+} (Figure 2M), approximately three times as

Table 1. Dependence of the Peptidase Activities of 7-DMATS, FgaPT1, CdpNPT, and FtmPT1 on the Presence of Different Additives with Final Concentration of 5 mM

	Relative Activity (%)				
Additive	7-DMATS	FgaPT1	CdpNPT	FtmPT1	FgaPT2
No additive	100 ± 4.1	100 ± 4.1	100 ± 0.8	100 ± 1.6	≤0.2
ATP	151.6 ± 4.4	145 ± 3.1	243.6 ± 5.8	149.4 ± 6.3	≤0.2
Ca^{2+}	102.7 ± 2.9	144.2 ± 8.5	69.2 ± 6.7	144.1 ± 2.3	≤0.2
Mg^{2+}	103.5 ± 1.2	137.9 ± 1.8	104.0 ± 6	155.2 ± 3.8	≤0.2
Mn^{2+}	84.0 ± 2.0	69.7 ± 1.1	591.2 ± 5.2	421.6 ± 1.7	≤0.2
Zn^{2+}	≤0.2	≤0.2	≤0.2	≤0.2	≤0.2
Co^{2+}	10.2 ± 1.3	32.3 ± 0.3	46.9 ± 1.5	130.4 ± 2.4	≤0.2
Fe^{2+}	≤0.2	≤0.2	34.2 ± 2.8	42.8 ± 0.5	≤0.2
Na^{+}	97.0 ± 3.9	108.2 ± 5.1	94.8 ± 0.8	108.6 ± 2	≤0.2
K^{+}	103.7 ± 2.7	117.9 ± 5.9	98.7 ± 4.3	86.5 ± 4.3	≤0.2
EDTA	11.6 ± 0.2	43.1 ± 2.1	≤0.2	≤0.2	≤0.2
ATP+Mn^{2+}	137.1 ± 0.8	140 ± 3.3	702.9	431.4 ± 1.2	≤0.2

In the cases of 7-DMATS and FtmPT1, H-L-Trp-Gly-OH was incubated with 0.6 μM protein. In the case of CdpNPT, FgaPT1, and FgaPT2, H-L-Trp-Gly-OH was incubated with 0.3 μM protein. All assays were incubated at 37°C for 45 min. The absolute conversion rates without additives were found to be 60.0%, 11.7%, 4.2%, and 2.9% for 7-DMATS, FgaPT1, CdpNPT, and FtmPT1, respectively.

that with Ca^{2+}. These results prompted us to reinvestigate the peptidase activity of FgaPT2 in the presence of different metal ions as used for 7-DMATS, FgaPT1, CdpNPT, and FtmPT1. In addition, experiments were carried out with Mn^{2+} at a final concentration from 2 mM to 50 mM. No peptidase activity could be observed for FgaPT2 under these conditions (Table 1).

Some peptidases or proteases are ATP-dependent (Chandu and Nandi, 2004). Addition of ATP to the enzyme assays increased the peptidase activities of 7-DMATS, FgaPT1, CdpNPT and FtmPT1 to 152, 145, 243, and 149%, respectively, in comparison to those without additives (Table 1). The peptidase activities of CdpNPT and FtmPT1 could be increased by a combination of ATP and Mn^{2+} to 703 and 431% of those without additives, respectively (Table 1).

In summary, the peptidase activities of the indole prenyltransferases FtmPT1 and CdpNPT were Mn^{2+}-dependent and could be enhanced by addition of ATP. For 7-DMATS and FgaPT1, enzymatic activity could be enhanced by addition of ATP, but not or only slightly enhanced by addition of metal ions such as Ca^{2+} or Mg^{2+}. However, based on their behavior in the presence of EDTA, it can be speculated that metal ions play also an important role for their peptidase activities.

Substrate Specificities and Kinetic Parameters of 7-DMATS, FgaPT1, CdpNPT, and FtmPT1 as Peptidases

To test and compare the substrate specificities of 7-DMATS, FgaPT1, CdpNPT, and FtmPT1, 31 peptides were incubated with 0.6 μM monomeric 7-DMATS and FtmPT1 and with 0.3 μM dimeric FgaPT1 and CdpNPT, respectively. The assays for FtmPT1 and CdpNPT contained Mn^{2+} at a final concentration of 5 mM and the assays for 7-DMATS and FgaPT1 contained Ca^{2+} at a final concentration of 5 mM. The tested peptides included 17 linear dipeptides, 8 tryptophan-containing cyclic dipeptides, 3 tryptophan-containing linear tripeptides, 2 tryptophan-containing linear tetrapeptides, and 1 tryptophan-containing linear hexapeptide (Table 2). After incubation at 37°C for 45 min, the reaction mixtures were analyzed on HPLC and the product formation was determined by quantification of tryptophan. The identity of the enzymatic products was confirmed by LC-MS analysis (data not shown).

Our results showed that all linear peptides with a tryptophanyl moiety at N terminus, i.e., with a free amino group at tryptophanyl moiety, were accepted by all of the enzymes. All of linear dipeptides with a tryptophanyl moiety at C terminus, i.e., with a free carboxyl group at tryptophanyl moiety, were not accepted under the conditions tested in this study. In general, accepted linear dipeptides were significantly better substrates for all of the enzymes than peptides with three or more amino acids. An exception was the tetrapeptide amide H-L-Trp-L-Met-L-Asp-L-Phe-NH_2, which was better accepted by CdpNPT and FtmPT1 than some linear dipeptides (Table 2). High conversion rates were determined for H-L-Trp-L-Ala-OH and H-L-Trp-Gly-OH with a product formation of 179.1 and 151.6 mmol * mol protein^{-1} * s^{-1} for 7-DMATS, 45.6 and 65.6 mmol * mol protein^{-1} * s^{-1} for FgaPT1, 49.4 and 69.8 mmol * mol protein^{-1} * s^{-1} for FtmPT1, as well as 179.4 and 162.2 mmol * mol protein^{-1} * s^{-1} for CdpNPT (Table 2). By comparison of the product formation under the respective best condition, it is also obvious that 7-DMATS and CdpNPT showed comparable enzymatic activities with H-L-Trp-L-Ala-OH as the best substrate. FgaPT1 and FtmPT1 accepted H-L-Trp-Gly-OH and H-L-Trp-L-Phe-OH as the best substrate, respectively, and showed a relative activity of 36% and 46% to those of 7-DMATS and CdpNPT by using H-L-Trp-L-Ala-OH as substrate, respectively. Linear dipeptides lacking a tryptophanyl moiety such as H-Gly-L-Tyr-NH_2, H-L-Tyr-Gly-OH H-L-Phe-L-Ala-OH, and H-L-His-L-Leu-OH were accepted by none of the enzymes. Tryptophan-containing cyclic dipeptides were also not substrates of the tested enzymes. These results demonstrated clearly that the indole prenyltransferases function also as tryptophan aminopeptidases and accepted linear dipeptides as best substrates.

Table 2. Peptidase Activities of 7-DMATS, FgaPT1, CdpNPT, and FtmPT1

Peptides	Product Formation (mmol product · mol protein^{-1} · s^{-1}) 7-DMATS	FgaPT1	CdpNPT	FtmPT1
H-L-Trp-L-Ala-OH	179.1	45.6	179.4	49.4
H-L-Ala –L-Trp-OH	≤0.5	≤0.5	≤0.5	≤0.5
H-L-Trp-Gly-OH	151.6	65.6	162.2	69.8
H-Gly-L-Trp-OH	≤0.5	≤0.5	≤0.5	≤0.5
H-L-Trp-L-Lys-OH	112.5	28.8	37.4	40.4
H-L-Lys –L-Trp-OH	≤0.5	≤0.5	≤0.5	≤0.5
H-L-Trp-L-Phe-OH	89.5	16.1	142.4	83.4
H-L-Trp-L-Asp-OH	58.0	14.6	38.5	49.0
H-L-Trp-L-Glu-OH	49.9	19.5	51.9	34.4
H-L-Glu-L-Trp-OH	≤0.5	≤0.5	≤0.5	≤0.5
H-L-Trp-L-Pro-OH	1.6	≤0.5	≤0.5	≤0.5
H-L-Pro-L-Trp-OH	≤0.5	≤0.5	≤0.5	≤0.5
H-L-Arg –L-Trp-OH	≤0.5	≤0.5	≤0.5	≤0.5
H-Gly-L-Tyr-NH_2	≤0.5	≤0.5	≤0.5	≤0.5
H-L-Tyr-Gly-OH	≤0.5	≤0.5	≤0.5	≤0.5
H-L-Phe-L-Ala-OH	≤0.5	≤0.5	≤0.5	≤0.5
H-L-His-L-Leu-OH	≤0.5	≤0.5	≤0.5	≤0.5
H-L-Trp-L-Trp-L-Trp-OH	3.3	≤0.5	8.1	47.6
H-Gly-L-Trp-Gly-OH	3.9	≤0.5	20.3	≤0.5
H-L-Leu-L-Trp-L-Leu-OH	1.1	≤0.5	≤0.5	≤0.5
H-L-Trp-Gly-Gly-L-Tyr	5.2	≤0.5	≤0.5	37.2
H-L-Trp-L-Met-L-Asp-L-Phe-NH_2	2.9	≤0.5	85.3	48.2
H-L-Trp-L-Thr-L-Val-L-Pro-L-Thr-L-Ala-OH	3.0	≤0.5	14.1	≤0.5
cyclo-L-Trp-L-Pro	≤0.5	≤0.5	≤0.5	≤0.5
cyclo-D-Trp-L-Pro	≤0.5	≤0.5	≤0.5	≤0.5
cyclo-Gly-L-Trp	≤0.5	≤0.5	≤0.5	≤0.5
cyclo-L-Leu-L-Trp	≤0.5	≤0.5	≤0.5	≤0.5
cyclo-L-Phe-L-Trp	≤0.5	≤0.5	≤0.5	≤0.5
cyclo-L-Trp-L-Trp	≤0.5	≤0.5	≤0.5	≤0.5
cyclo-L-Trp-L-Tyr	≤0.5	≤0.5	≤0.5	≤0.5
cyclo-D-Trp-L-Tyr	≤0.5	≤0.5	≤0.5	≤0.5

Assays of 7-DMATS and FgaPT1 contained $CaCl_2$ at a final concentration of 5 mM and assays of CdpNPT and FtmPT1 contained $MnCl_2$ at a final concentration of 5 mM.

Linear tri-, tetra-, and hexapeptides were also substrates of these enzymes with relatively lower conversion rates (with the exception of H-L-Trp-L-Met-L-Asp-L-Phe-NH_2, mentioned above), in comparison to linear dipeptides with a free amino group at the tryptophanyl moiety. Interestingly, H-Gly-L-Trp-Gly-OH, H-L-Leu-L-Trp-L-Leu-OH with a tryptophanyl moiety linked directly to two amino acids by two amide bonds were also accepted by the enzymes. This phenomenon was also observed for other aminopeptidases; e.g., leucine aminopeptidase LAP-A of potato also accepted tripeptides (Gu and Walling, 2000).

For a better understanding of this enzyme group, kinetic parameters of the peptidase activities were determined by using H-L-Trp-L-Gly-OH as substrate and compared with those of their prenyltransferase activities (Table 3). The peptidase reactions of 7-DMATS, FgaPT1, CdpNPT, and FtmPT1 followed Michaelis-Menten kinetics. Comparable K_m values for the peptidase activities were found to be 350 μM, 380 μM, 300 μM, and 420 μM for 7-DMATS, FgaPT1, CdpNPT, and FtmPT1, respectively. These values are significantly higher than those of the prenyltransferase activities in the presence of DMAPP; e.g., 137 μM for 7-DMATS by using L-tryptophan as substrate (Kremer et al., 2007), 6 μM for FgaPT1 by using fumigaclavine A as substrate (Unsöld and Li, 2006), 55 μM for FtmPT1 by using brevianamide F as substrate (Grundmann and Li, 2005), as well as 128 μM for CdpNPT by using cyclo-D-Trp-L-Tyr in the presence of DMAPP (W.-B. Yin and S.-M.L., unpublished data; Table 3). Higher turnover numbers (k_{cat}) were also found for the prenyltransferase activities than for the peptidase activities (Table 3). As consequence, much higher enzymatic rate constants (k_{cat} K_m^{-1}) were determined for the prenyltransferase activities than for the peptidase activities. The enzymatic rate constants for the peptidase activities were found to be comparable in the range of 0.14 to 0.53 $mM^{-1}s^{-1}$. In contrast, the values for the prenyltransferase activities varied from 1.68 $mM^{-1}s^{-1}$ for 7-DMATS to 133.33 $mM^{-1}s^{-1}$ for FgaPT1 (Table 3).

DISCUSSION

In this study, we demonstrated that the indole prenyltransferases 7-DMATS, FgaPT1, FtmPT1, and CdpNPT from *Aspergillus fumigatus* showed significant tryptophan aminopeptidase activities toward linear dipeptides. To the best of our knowledge, this is the first report on the catalytic promiscuity of prenyltransferases. The chemical transformations of both reactions differ not only in the involved functional groups, but also in the reaction mechanisms, i.e., forming of a C-C bond versus breaking of a C-N bond. Catalytic promiscuity in enzymes was observed with different proteins and plays a natural role in evolution and in the biosynthesis of natural products (Bornscheuer and Kazlauskas, 2004). The genes coding for the enzymes described in this study belong to different biosynthetic gene clusters of secondary metabolites and their function as prenyltransferases have been unequivocally proven by biochemical characterizations (Grundmann and Li, 2005; Kremer et al., 2007; Li and Unsöld, 2006; Ruan et al., 2008; Unsöld and Li, 2006; Yin et al., 2007). L-tryptophan is the substrate of the prenyltransfer reaction of 7-DMATS (Kremer et al., 2007) and tryptophan-containing cyclic dipeptides were accepted as substrates by FtmPT1 (Grundmann and Li, 2005) and CdpNPT (Ruan et al., 2008), while a somewhat complicated structure fumigaclavine A is the natural substrate for FgaPT1 (Unsöld and Li, 2006; Figure 1). A common feature of these substrates is the indole moiety in tryptophan or structures derived thereof. Interestingly, the second dimethylallyltryptophan synthase FgaPT2 involved in the biosynthesis of fumigaclavines (Unsöld and Li, 2005) showed no aminopeptidase activity under conditions described in this study. From our results, it can not be determined whether and which roles the aminopeptidase activities could play for the prenyltransfer reactions and/or for the biosynthesis of the respective secondary metabolites. No direct correlation could be found between prenyltransfer reactions and aminopeptidase activities. For

Table 3. K_m Values and Turnover Numbers of Prenyltransferase and Peptidase Activities of Recombinant Enzymes

	Prenyltransferase Activity			Peptidase Activity		
Enzyme	K_m [mM]	k_{cat} [s^{-1}]	k_{cat}/K_m [$mM^{-1}s^{-1}$]	K_m [mM]	k_{cat} [s^{-1}]	k_{cat}/K_m [$mM^{-1}s^{-1}$]
7-DMATS	0.137	0.23	1.68	0.35	0.18	0.51
FgaPT1	0.006	0.80	133.33	0.38	0.09	0.24
CdpNPT	0.128	0.46	3.59	0.30	0.16	0.53
FtmPT1	0.055	5.57	101.27	0.42	0.06	0.14
FgaPT2	0.009	0.27	30.00	-	-	-

example, the natural substrates of the prenyltransfer reactions of 7-DMATS and FgaPT1 are L-tryptophan and fumigaclavine A, respectively, which lack a peptide structure as substrates for the peptidase activities. Tryptophan-containing cyclic dipeptides were no substrates for the peptidase activities of the prenyltransferases but they could be prenylated by CdpNPT and FtmPT1 in the presence of DMAPP (Grundmann and Li, 2005; Ruan et al., 2008). In case of 7-DMATS, however, it could be speculated that both peptidase and prenyltransferase activity could be involved in the biosynthesis of the same secondary metabolite. *7-dmats* belongs to a putative biosynthetic gene cluster containing a gene coding for a putative nonribosomal peptide synthetase (NRPS) EAL92291. The product of the gene cluster could be a derivative of a cyclic dipeptide containing 7-DMAT (Kremer et al., 2007). We have proposed that L-tryptophan could be at first converted to 7-DMAT catalyzed by the prenyltransferase 7-DMATS. 7-DMAT would then undergo the condensation with a second amino acid, which could not be predicted by the sequence analysis, to a prenylated (cyclic) dipeptide under the catalysis of EAL92291 (Kremer et al., 2007). Like the NRPS GliP in the biosynthesis of gliotoxin consisting of L-Phe and L-Ser, EAL92291 has a trimodular architecture (A1-T1-C1-A2-T2-C2-T3) (Balibar and Walsh, 2006). It has been proven by biochemical investigation that the A1 and A2 domains in GliP are responsible for the activation of L-Phe and L-Ser, respectively, which are then loaded onto the T1 and T2 domains of GliP, respectively. The linear enzyme-bound peptide L-Phe-L-Ser-T2 was formed under the catalysis of the condensation domain C1 (Balibar and Walsh, 2006). The roles of the second condensation domain C2 and the third thiolation domain T3 are unclear, although the mutational experiments suggested that they are involved in the same process (Balibar and Walsh, 2006). It was speculated that the subsequent reactions to gliotoxin, e.g., methylation or sulfur insertion, may occur while the linear dipeptide is still covalently bound to the NRPS GliP (Balibar and Walsh, 2006). The release of an intermediate in the biosynthesis could occur at some later step. This could also be the case for EAL92291. The peptidase activity of 7-DMATS could be involved in the release of the dipeptide from the NRPS EAL92291, because aminopeptidases often have esterase activity (Bornscheuer and Kazlauskas, 2004).

Furthermore, aminopeptidase activities of the indole prenyltransferases could be involved in peptide degradation and pathogenesis of their natural producer, i.e., *Aspergillus fumigatus*. Aminopeptidases perform essential cellular functions, including many normal and pathophysiological processes, and are widely distributed among bacteria, fungi, plants, and mammals (Gonzales and Robert-Baudouy, 1996; Lowther and Matthews, 2002; Taylor, 1993). Intracellular proteolytic degradation is important for the elimination of damaged proteins, the modulation of protein levels, and the maintenance of amino acid pools (Basten et al., 2001). In bacteria and fungi, aminopeptidases are involved in the utilization of exogenous peptides as nutrients after degradation with a variety of peptidases of different specificity (Gonzales and Robert-Baudouy, 1996). For the uptake of short chain peptides, *Aspergillus* species have not been reported to have many transport systems, and, therefore, the proteolytic enzymes should contain a range of different peptidases to enable the organism to degrade the various proteins to free amino acids (Garraway and Evans, 1984; Nampoothiri et al., 2005). Different aminopeptidases have been identified in *Aspergillus* species including leucine, lysine, phenylalanine, aspartate, and proline, but no tryptophan aminopeptidase (Basten et al., 2001, 2003, 2005; Chien et al., 2002; Nampoothiri et al., 2005; Watanabe et al., 2007). A literature search revealed only one known tryptophan aminopeptidase (EC 3.4.11.17) from the yeast *Trichosporon cutaneum* (Iwayama et al., 1983), although 2000 peptidase species have been characterized from different sources (Barrett and Rawlings, 2007). Therefore, it can be speculated that the degradation of tryptophan-containing peptides for primary and secondary metabolism is carried out by other aminopeptidases with broad substrate specificity or even by other enzyme groups, e.g., prenyltransferases reported in this study. The part of tryptophan released by indole prenyltransferases could be directly used without any transport process through different cell compartments for the biosynthesis of the respective tryptophan-containing natural products.

Aspergillus fumigatus produces during its mycelial growth different toxic metabolites such as gliotoxin and enzymes including different proteases, which could play a role in pathogenesis (Rementeria et al., 2005; Tomee and Kauffman, 2000). Although none of these factors has been shown to be involved in the pathogenesis of *Aspergillus fumigatus* in experimentally induced infections (Brakhage, 2005; Hohl and Feldmesser, 2007; Latgé, 2001), their involvement in the pathogenesis cannot be excluded. All data accumulated to date suggest that many factors are involved in the mycelial virulence of *Aspergillus fumigatus* (Hohl and Feldmesser, 2007; Latgé, 2001). It could be speculated that indole prenyltransferases are involved with their peptidase activities in the pathogenesis directly in the case of FgaPT1 and 7-DMATS or after induction by metal ions, e.g., Mn^{2+}, in the case of FtmPT1 and CdpNPT. As mentioned above, peptide degradation would supply amino acids, which are essential for the fungal growth and development.

A database search revealed no sequence similarity of the indole prenyltransferases to known proteases or peptidases.

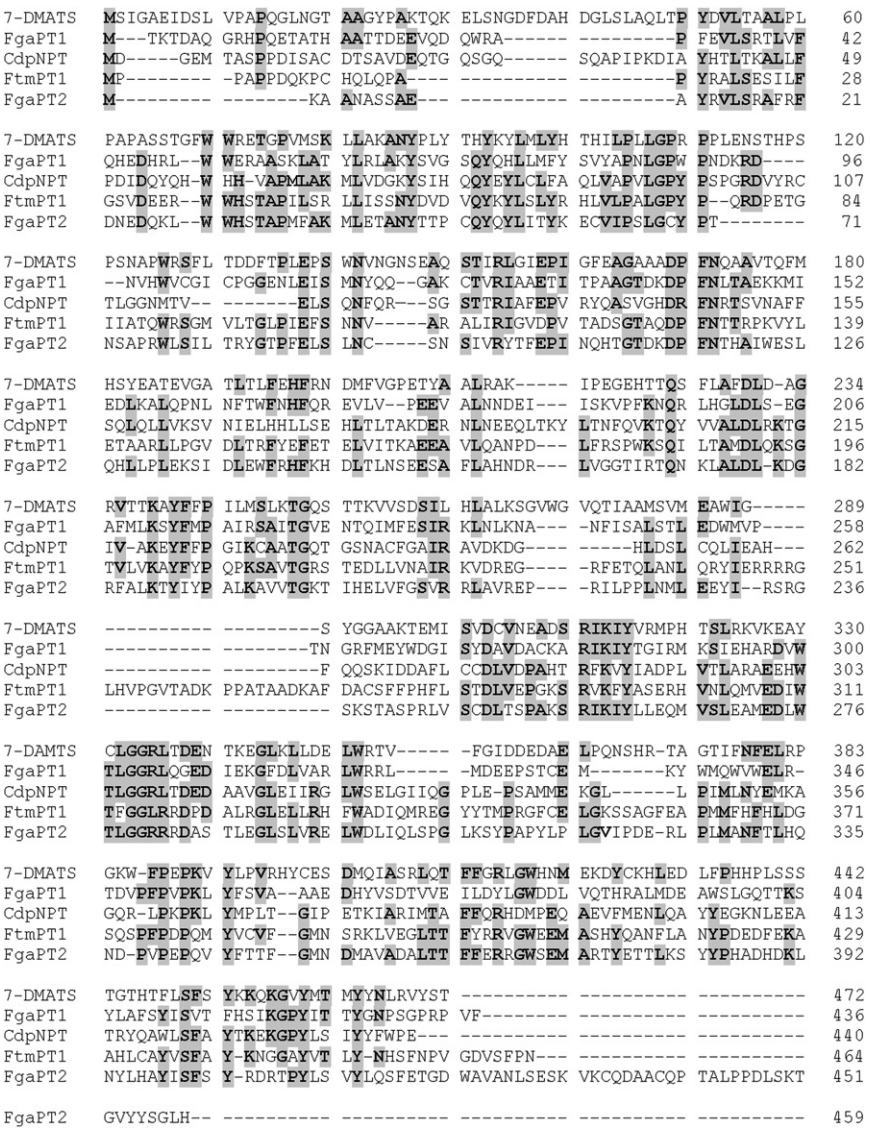

Figure 3. Alignments of Known Prenyltransferases from *Aspergillus fumigatus*

Sequence analysis of 7-DMATS, FgaPT1, CdpNPT, and FtmPT1 showed no conserved motifs for known metal binding sites, e.g., those found in a leucine aminopeptidase from *Aspergillus sojae* (Chien et al., 2002). Comparison of the sequences of 7-DMATS, FgaPT1, CdpNPT, and FtmPT1 with that of FgaPT2 (Figure 3) indicated no significant difference of motifs, which could be responsible for their different behavior regarding the peptidase activities.

The indole prenyltransferases 7-DMATS, FgaPT1, CdpNPT, and FtmPT1 showed high substrate specificities for their aminopeptidase activities regarding the position of the tryptophanyl moiety in the peptide molecules. As shown in this study, tryptophan-containing linear dipeptides with an N-terminal tryptophan moiety, e.g., H-L-Trp-L-Ala-OH, H-L-Trp-Gly-OH, and H-L-Trp-L-Lys-OH, were well accepted as substrates for the aminopeptidase activities. The counter partners of these linear dipeptides, H-L-Ala–L-Trp-OH, H-Gly-L-Trp-OH, or H-L-Lys-L-Trp-OH, were not accepted (Table 2). This feature differed clearly from many known aminopeptidases from bacteria and fungi, which showed higher substrate flexibility (Chien et al., 2002; Gonzales and Robert-Baudouy, 1996; Gu and Walling, 2000; Herrera-Camacho et al., 2000; Monod et al., 2005). Aminopeptidases belong to the large group of metallopeptidases and their reactions are dependent on divalent metal ions such as Zn^{2+}, Mg^{2+}, and Mn^{2+} (Gonzales and Robert-Baudouy, 1996; Lowther and Matthews, 2002; Taylor, 1993). The most investigated aminopeptidases are leucine, lysine, cysteine, serine, and methionine aminopeptidases (Gonzales and Robert-Baudouy, 1996; Lowther and Matthews, 2002; Nampoothiri et al., 2005). The tryptophan aminopeptidase from *Trichosporon cutaneum* mentioned above (Iwayama et al., 1983) showed broad substrate specificity toward linear dipeptides and accepted also different simple amino acid amides as substrates (Iwayama et al., 1983). Mn^{2+} was essential for the enzymatic reaction of the aminopeptidase from *Trichosporon cutaneum* (Iwayama et al., 1983), which is consistent with other known aminopeptidases.

Correspondingly, the aminopeptidase activities of 7-DMATS, FgaPT1, CdpNPT, and FtmPT1 are dependent on the presence of metal ions. Strong or complete inhibition of the peptidase activities was observed after addition of EDTA to the reaction mixtures (Table 1), indicating the importance of metal ions, which were removed by EDTA. Direct support for this speculation was provided by addition of Mn^{2+} to the reaction mixtures of FtmPT1 and CdpNPT. The peptidase activities could be increased up to 4- and 6-fold for FtmPT1 and CdpNPT, respectively (Table 1). These data proved that Mn^{2+} ions are involved in the peptidase reaction of the indole prenyltransferases, at least for those of FtmPT1 and CdpNPT. Addition of Mn^{2+} to the reaction mixtures of 7-DMATS and FgaPT1 reduced the peptidase activities to 84% and 70% of those without additives, respectively. Ca^{2+} and Mg^{2+} enhance the peptidase activities of FgaPT1 and FtmPT1 slightly. It is plausible that 7-DMATS and FgaPT1 bind metal ions more tightly than FtmPT1 and CdpNPT and contain enough metal ions for their peptidase activities, even after protein purification on Ni-NTA agarose. These metal ions could be partly or completely removed by addition of EDTA, resulting in decreasing of peptidase activities. Supports for this explanation were provided by the fact that 7-DMATS and FgaPT1 showed higher peptidase activities without additives than FtmPT1 and CdpNPT. The conversion rates of H-L-Trp-Gly-OH without

additives were found to be 60.0%, 11.7%, 4.2%, and 2.9% for 7-DMATS, FgaPT1, CdpNPT, and FtmPT1, respectively. Considering the different enzyme amounts in the assays (0.6 μM 7-DMATS and FtmPT1, 0.3 μM FgaPT1 and CdpNPT), relative peptidase activities of 100%, 39.0%, 14.0%, and 4.8% could be calculated from these data for 7-DMATS, FgaPT1, CdpNPT, and FtmPT1, respectively. After addition of Mn^{2+} to the reaction mixtures, the enzyme activities of FtmPT1 and CdpNPT could be reached to 422% and 591% of those without additives, respectively.

The dependence of the amiopeptidase activities of indole prenyltransferases on metal ions is sharply in contrast to their prenyltransferase activities. No divalent metal ions are essential for the prenyltransferase reactions, although Ca^{2+} enhances the enzyme activities. No or nearly no loss of the prenyltransferase activities was observed after addition of EDTA to the reaction mixtures of all of the purified and characterized prenyltransferases (Grundmann and Li, 2005; Kremer et al., 2007; Li and Unsöld, 2006; Unsöld and Li, 2006; Yin et al., 2007). Similar phenomenon was also observed with TdiB, an indole prenyltransferase from *Aspergillus nidulans* (Schneider et al., 2008). The requirements of the prenyltransfer and hydrolysing reactions on metal ions could indicate different active sites of the enzymes for both reactions. However, it was also reported that catalytic promiscuity could be created by binding of different metal ions at the same active site (Bornscheuer and Kazlauskas, 2004). Site-directed mutagenesis or protein structure would provide information on the active sites of the prenyltransferase and peptidase activities of the indole prenyltransferases from fungi.

We have speculated that the indole prenyltransferases from fungi have evolved directly from a common ancestor (Kremer et al., 2007; Yin et al., 2007). Our results reported here provide further support for this hypothesis. Divergent evolution is a natural process that creates different species from a common ancestor, which is also applicable for creation of enzymes with new catalytic activities. New enzymatic activities arise by gene duplication, followed by evolution of new activity for the copy (Bornscheuer and Kazlauskas, 2004; Gerlt and Babbitt, 2001). It could be speculated that an earlier protein carried only one enzymatic activity and the second one was created after gene duplication. It cannot be proposed, however, which activity was the first enzymatic activity of this enzyme group. From the much higher catalytic efficiencies of the prenyltransferase activities in comparison to those of peptidase activities (Table 3), on the other hand, it could be postulated that the prenyltransferase activities were present before the peptidase activities. During the evolution, some enzymes adapted the second catalytic activity, namely, the peptidase activity (e.g., 7-DMATS, FgaPT1, FtmPT1 and CdpNPT described in this study). Others have still only prenyltransferase activities (e.g., FgaPT2). It seems that specifications for the prenyltransferase activities have also taken place during the evolution. The substrate specificities and enzymatic rate constants of the prenyltransfer reactions catalyzed by these enzymes differed clearly from each other. On the other hand, these parameters are similar for their peptidase activities. But the peptidase activities of some enzymes, e.g., CdpNPT and FtmPT1, can be induced at least in vitro by metal ions such as Mn^{2+}. It would be interesting to investigate the aminopeptidase activities of other prenyltransferases from fungi, plants, or bacteria.

Peptidases are widely used in research and diagnostics and in the leather, food, and pharmaceutical industries (Rao et al., 1998), e.g., for post-hydrolysis of protein hydrolysates. Protein hydrolysates are acidic, alkaline, or enzymatic hydrolysis products of proteins; contain short-chain peptides and free amino acids; and are used as constituents of dietetic and health products, clinical nutrition supplements, or as flavoring agents (Clemente, 2000; Rao et al., 1998). The bitter taste of protein hydrolysates is a major barrier for their use in food and health care products (Clemente, 2000; Rao et al., 1998). The intensity of the bitterness is proportional to the number of hydrophobic amino acids in the hydrolysates (Clemente, 2000; Ishibashi et al., 1988; Rao et al., 1998). Exopeptidases such as aminopeptidases can reduce the amount of peptides with undesirable tastes through the removal of a single hydrophobic amino acid, or pairs of them, from the terminal ends and are therefore used in the posthydrolysis processes in the production of protein hydrolysates (Clemente, 2000; Rao et al., 1998). Tryptophan is the most hydrophobic of amino acid side chains (Nozaki and Tanford, 1971) and tryptophan-containing peptides therefore also contribute to the bitterness of protein hydrolysates (Lalasidis, 1978). For this reason, tryptophan aminopeptidase could find application for debittering of protein hydrolysates by removal of N-terminal tryptophan moiety. In analogy to many commercial aminopeptidases, a further possible application of the enzymes described in this study could be for determination of tryptophan-containing peptides, especially dipeptides.

SIGNIFICANCE

The results described in this study showed that four of five identified indole prenyltransferases carried also tryptophan aminopeptidase activities. To the best of our knowledge, this is the first report on the catalytic promiscuity of prenyltransferases. Similar substrate specificity was observed for the peptidase activities, while different indole derivatives were substrates of the prenyltransfer reactions. The aminopeptidase activities of all of the four enzymes could be inhibited by addition of EDTA. In addition, the aminopeptidase activities of FtmPT1 and CdpNPT were strictly dependent on the presence of Mn^{2+} ions and were strongly enhanced by addition of Mn^{2+} to the reaction mixtures. This is in contrast to the prenyltransfer reactions catalyzed by these enzymes, which are independent of the presences of metal ions. The results described in this study provide further supports for the hypothesis that the indole prenyltransferases from fungi have evolved directly from a common ancestor.

EXPERIMENTAL PROCEDURES

Chemicals

The synthesis of brevianamide F has been reported previously (Grundmann and Li, 2005). The other peptides were purchased from Bachem.

Protein Overproduction and Purification

Overproduction and purification of His_6-7-DMATS, His_6-FgaPT1, His_6-CdpNPT, His_6-FtmPT1, and His_8-FgaPT2 were described previously (Grundmann and Li, 2005; Kremer et al., 2007; Steffan et al., 2007; Unsöld and Li, 2006; Yin et al., 2007).

Determination of Molecular Weight of Native Enzymes

The molecular weights of native His_6-FgaPT1 and His_6-FtmPT1 were reported previously (Grundmann and Li, 2005; Unsöld and Li, 2006).

The molecular weights of native His_6-7-DMATS and His_6-CdpNPT were determined by gel filtration on a HiLoad 16/60 Superdex 200 column (GE Health Care) which had been equilibrated with 50 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl. The column was calibrated with dextran blue 2000 (2000 kDa), conalbumin (75 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), ribonuclease A (13.7 kDa), and aprotinin (6.5 kDa) (GE Healthcare). The proteins were eluted with 50 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl. The molecular weights of native His_6-7-DMATS and His_6-CdpNPT were determined as 65 kDa and 104 kDa, respectively. This proved that His_6-7-DMATS acts as a monomer and His_6-CdpNPT acts as a homodimer.

Assay for Peptidase Activity

For quantitative determination of the enzyme activity of 7-DMATS and FtmPT1, the reaction mixture (100 μl) contained 50 mM Tris-HCl (pH 7.5), 2 mM aromatic substrates, 1.5% (v/v) glycerol, 0.6 μM purified recombinant proteins, and 5 mM $CaCl_2$ in the case of 7-DMATS (5 mM $MnCl_2$ in the case of FtmPT1). For quantitative determination of the enzyme activity of FgaPT1 and CdpNPT, the reaction mixture (100 μl) contained 50 mM Tris-HCl (pH 7.5), 2 mM aromatic substrates, 1.5% (v/v) glycerol, 0.3 μM purified recombinant proteins, and 5 mM $CaCl_2$ in the case of FgaPT1 (5 mM $MnCl_2$ in the case of CdpNPT). After incubation for 45 min at 37°C, the reaction was stopped with 100 μl methanol. The protein was removed by centrifugation at 13,000 × g for 10 min. The enzymatic products were analyzed by HPLC under conditions described below. For quantitative measurement of the enzyme activity, duplicate values were determined routinely.

HPLC Analysis

The enzymatic products of the incubation mixtures were analyzed by HPLC on an Agilent HPLC Series 1100 by using an Eclipse XDB-C18 column (4.6 × 150 mm, 5 μm [Agilent]) at a flow rate of 1 ml min^{-1}. For separation, a linear gradient of 30%–70% (v/v) methanol containing 0.5% (v/v) aqueous trifluoroacetic acid (TFA) in 15 min was used. The column was then washed with 100% methanol containing 0.5% (v/v) TFA for 5 min and equilibrated with 30% (v/v) methanol containing 0.5% (v/v) TFA for 5 min. Detection was carried out by a Photo Diode Array detector.

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