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Unveiling the biosynthetic pathway of the representative ribosomal peptide ustiloxin B in filamentous fungi

Ying Ye, Atsushi Minami, Yuya Igarashi, Miho Izumikawa, Myco Umemura, Nozomi Nagano, Masayuki Machida, Teppei Kawahara, Kazuo Shin-ya, Katsuya Gomi, and Hideaki Okawa*

Abstract: The biosynthetic machinery of the first fungal ribosomal peptide ustiloxin B was elucidated through a series of gene inactivation and heterologous expression studies. The results confirmed the essential requirement of novel oxidases possessing the DUF3328 motif for macrocyclization and highly unique side chain modifications by three oxidases (UstCF1F2) and a PLP-dependent enzyme (UstD). These findings provide a new insight for biosynthesis of ribosomal peptide gene clusters found in various fungi.

Ustiloxins represented by B (1) were isolated as phytotoxins from rice false smut caused by the pathogenic fungus Ustilaginoidea virens (Figure 1).[1-3] These family members, including a structurally related phomopsin (Figure 1), exhibit potent antimitic activity and inhibit microtubule assembly.[2] Recently, a biosynthetic gene cluster (ust) of 1 was identified in the genome of Aspergillus flavus using a sequence motif-independent de novo detection algorithm (MIDDAS-M) for secondary metabolite gene clusters.[4,5] Ribosomally synthesized and post-translationally modified peptides (RiPPs) are often found in the genomes of various species of bacteria.[6] In contrast, the ustiloxin gene cluster was the first example of an RiPP from the filamentous fungi, while the precursor peptide gene for α-amanitin was identified in mushrooms (Figure 1).[7] The cluster contained a unique precursor protein, UstA, that has an unprecedented 16 repeated core peptide regions. After identification of the ust cluster, we found more than 94 homologous clusters in the Aspergilli genome sequences.[8] Very recently, gene clusters of new ribosomal peptides, epichloëycclin and asperipin-2a, have been reported.[8,9] In this communication, we report the complete characterization of the core peptide formation with three oxidation enzymes uniquely found in the gene clusters of the filamentous fungal RiPPs and the subsequent modifications specific for ustiloxin family biosynthesis.

On the basis of previous gene knockout experiments, we proposed that 11 genes were responsible for ustiloxin biosynthesis.[8] Detailed LC-MS analysis of the extracts from five mutants, AустM, AустC, AустF1, AустF2, and AустD, showed the accumulation of ustiloxin derivatives (2-6) with characteristic UV spectra (Figure 2, Figure S1). Based on HR-MS data, the molecular formulae of the compounds were deduced as follows: 2: C20H32N4O8; 3: C21H30N4O8; 4: C24H35N5O10S; 5: C24H34N4O10S; and 6: C23H34N4O10S. Among these metabolites, 3 and 6 were readily identified as ustiloxin F and ustiloxin C, respectively, from the 1H-NMR data.[2,3] Comparison of the 1H-NMR spectra of 2 and 3 indicated that these were closely related and that the N-methyl signal (δH 2.74) in the spectrum of 3 was not present in that of 2, suggesting that 2 is a desmethyl derivative of 3. The higher molecular weights of 4/5 compared to 3 indicated the addition of a cysteine residue (C2H2NSO2/C2H2NSO3). In the NMR spectra of 4 and 5, the lack of one of the aromatic proton (3: δH 7.04) present in 3 indicated the substitution by a cysteine residue. Additionally, the signal patterns were nearly identical, and the shifted signals were observed only at the Tyr side chain (4: δH 3.43, 3.61 (CH3); 5: δH 3.15, 3.71 (CH3)) and the aromatic portion (4: δH 7.14, 7.27; 5: δH 7.63, 7.42), suggesting that 5 is a sulfide derivative of 4, which we named as ustiloxin H. The proposed structures of 2, 4, and 5 were supported by high-resolution MS and ESI-MS/MS data (Figure S2). The NMR spectra of 6 suggested that this compound is a sulfoxide derivative of 5. In addition, LC-MS analysis indicated that 1 is a desmethyl derivative of 2. The NMR spectra of 1 suggested that this compound is a sulfoxide derivative of 2. Additionally, the signal patterns were nearly identical, and the shifted signals were observed only at the Tyr side chain (6: δH 3.43, 3.61 (CH3); 7: δH 3.15, 3.71 (CH3)) and the aromatic portion (6: δH 7.14, 7.27; 7: δH 7.63, 7.42), suggesting that 7 is a sulfide derivative of 6, which we named as ustiloxin I. The proposed structures of 2, 4, and 5 were supported by high-resolution MS and ESI-MS/MS data (Figure S2).
were confirmed by extensive NMR analyses including COSY, HSQC, and HMBC. These data enabled us to speculate on the functions of the five ustMCF1F2D genes. For the last transformation with UstD, condensation of a C3 nucleophile with an aldehyde form of 6 is most likely to be involved. Taking into account these data, we proposed a biosynthetic pathway for 1 as shown in Scheme 1.

Figure 2. LC-MS profiles of the ustiloxin derivatives: (A) 2 produced by ustM, (B) 3 produced by ustC, (C) 4 produced by ustF1, (D) 5 produced by ustF2, (E) 6 produced by ustD.

Scheme 1. Proposed biosynthetic pathway of ustiloxin.

Figure 3. LC-MS profiles of extracts from transformants: (A) WT strain, (B) AO-ustAQYa, (C) AO-ustAQYb, (D) AO-ustAQYaYb (co-transformation), and (E) AO-ustAQYaYb (stepwise incorporation).
fungi, they may cause damage to the host. Our findings may aid in the heterologous production of various ribosomal peptides in a versatile host such as A. oryzae, which presents a wide range of tolerance against various antibiotics and metabolites that are toxic to eukaryotic cells.\[10,12\]

We then turned our attention to the functional analysis of Class B bifunctional flavoprotein monooxygenases,\[13\] UstF1 and UstF2, which participate in the side chain modifications of 4. Both purified maltose binding protein (MBP)-tagged enzymes showed a yellow color and strong absorption at 450 nm in the UV-visible spectra (Figure S3), indicating a tight binding of a FAD as is the case for other Class B flavoproteins. Oxidation of 4 with UstF1 was examined in the presence of NADPH. LC-MS analysis of the reaction mixtures showed a new peak corresponding to 5 (Figure 4A). Incubation of 5 with UstF2 and NADPH yielded two isomeric mixtures of oximes 7 (Figure 4B), whose molecular formula was determined to be C$_2$H$_{16}$N$_4$O$_4$S by HR-MS analysis. In the $^1$H-NMR spectrum of 7, the $\alpha$-proton of a Cys residue in 5 was missing (NMR spectra in the Supporting information), and new oxime methine signals appeared ($E$ isomer (major); $\delta_\mathrm{H}$ 7.36, $Z$ isomer (minor); $\delta_\mathrm{H}$ 6.89). Extensive NMR analyses confirmed the structure of 7. Treatment of 7 with 0.1% TFA readily afforded the corresponding geminal diol 9, a hydrate form of 8. During NMR measurement of 7 and 9 in D$_2$O, we observed relatively rapid H/D exchange of C2'-CH$_2$ in the side chain due to their acidity (Figure S4, NMR spectra in the Supporting information). Reduction of 9 with NaBH$_4$ yielded 6, which was identical to ustiloxin C from the ustid deletion mutant in all respects. To elucidate the formation mechanism of oxime 7 catalyzed by UstF2, a time course analysis was then performed (Figure S5). At the beginning of the reaction, a new peak of 10 was observed along with 7 in the LC-MS analysis. Its molecular weight ($\text{m/z}$ 618 [M+H]$^+$) strongly suggested that 10 is a monohydroxylated intermediate of 5. Based on these observations, we proposed a reaction mechanism involving two rounds of N-hydroxylation followed by decarboxylative dehydration to give 7 (Scheme 2A). Similar oxime formation was reported in caerulomycin A biosynthesis, which involves sequential N-hydroxylations with monooxygenase CrmH followed by dehydration instead of decarboxylative dehydration in the case of 7 (Scheme S1).[14]

**Figure 4.** LC-MS profiles of the enzymatic reaction products of (A) UstF1 reaction, (B) UstF2 reaction, (C) UstD reaction with alanine, and (D) UstD reaction with aspartic acid.

Considering that the last transformation is catalyzed by the PLP-dependent enzyme UstD, we speculated that a C3 nucleophile, such as an enamine derived from alanine or aspartate, likely reacts with a putative aldehyde intermediate 8 to give 1. Therefore, we incubated 9 with a MBP-tagged UstD in the presence of PLP and amino acids. In the case of aspartic acid, formation of 1 was observed (Figures 4C-4D), while in the absence of 9, we detected a peak of a dansylated alanine in the HPLC analysis after treatment of the reaction mixture with dansyl chloride (Figure S6). These results indicated that UstD catalyzed a decarboxylation of aspartate to yield an enamine followed by condensation with aldehyde 8 to give 1, as shown in Scheme 2B. Although the mechanism of aspartate $\beta$-decarboxylase has been established in various studies including X-ray crystal analysis (Scheme S2),\[15\] to our knowledge, condensation of the resultant enamine with an aldehyde in the same active site has not been reported before.

For the formation of the first cyclization product 2, three oxidation steps are required: i) hydroxylation at the benzylic position, ii) hydroxylation at either the aromatic ring of Tyr or $\beta$-position of Ile, and iii) oxidative cyclization. Based on the putative function of tyrosinase, UstQ may catalyze the oxidation of a phenol moiety, whereas the uncharacterized DUF3328 proteins UstYa and UstYb are most likely responsible for the remaining two-step oxidations. Very recently, a new ribosomal peptide, asperinpin-2a, possessing two macrocyclic ether rings was reported.[8] In its biosynthetic gene cluster consisting of four genes, the UstY homolog was found to be the sole oxidation enzyme. To date, only three gene clusters for the ribosomal peptides have been characterized.[13,8,9] However, a set of biosynthetic genes encoding a precursor peptide and UstY homolog have been identified in many fungal genomes.[8,9] This circumstantial evidence suggests that the most intriguing macrocyclization of the core peptide is catalyzed by a UstY homolog.

In summary, we unveiled the entire biosynthetic pathway of ustiloxin involving nine enzymes utilizing gene inactivation, heterologous expression, and in vitro functional analyses. In particular, the heterologous expression data were very useful for elucidating the key role of UstA/Q/Ya/Yb in the macrocyclic formation of ustiloxins, suggesting that this method can be applied for the functional analysis of fungal ribosomal peptides. In addition, functional analysis of the side chain modification enzymes revealed the unique oxidations of sulfur and nitrogen atoms (two homologous-FAD dependent enzymes, UstF1 and UstF2) and decarboxylative C-C bond formation (the PLP-dependent enzyme, UstD). Currently, the unprecedented oxidative cyclization of the core peptide catalyzed by UstQYaYb is under investigation.

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The biosynthetic machinery of fungal ribosomal peptide ustiloxin has been elucidated by gene inactivation, heterologous expression, and in vitro studies. These included the cyclic peptide formation catalysed by novel oxidases harbouring DUF3328 motif and unique modification reactions for construction of the side chain on the aromatic ring. These findings set a new stage for elucidating the fungal ribosomal peptide biosynthesis.