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Yousong Ding
University of Nebraska - Lincoln

Ravi S. Bojja
University of Nebraska - Lincoln

Liangcheng Du
University of Nebraska - Lincoln, ldu3@unl.edu

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Fum3p, a 2-Ketoglutarate-Dependent Dioxygenase Required for C-5 Hydroxylation of Fumonisins in *Fusarium verticillioides*

Yousong Ding, Ravi S. Bojja, and Liangcheng Du*

Chemistry Department, University of Nebraska—Lincoln, Lincoln, Nebraska 68588-0304

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Fumonisins are polyketide-derived mycotoxins produced by several agriculturally important *Fusarium* species. The B series fumonisins, FB_1, FB_2, FB_3, and FB_4, are fumonisins produced by wild-type *Fusarium verticillioides* strains, differing in the number and location of hydroxyl groups attached to the carbon backbone. We characterized the protein encoded by *FUM3*, a gene in the fumonisin biosynthetic gene cluster. The 33-kDa *FUM3* protein (Fum3p) was heterologously expressed and purified from *Saccharomyces cerevisiae*. Yeast cells expressing the Fum3p converted FB_3 to FB_1, indicating that Fum3p catalyzes the C-5 hydroxylation of fumonisins. This result was verified by assaying the activity of Fum3p purified from yeast cells. The C-5 hydroxylase activity of purified Fum3p required 2-ketoglutarate, Fe^{2+}, ascorbic acid, and catalase, all of which are required for 2-ketoglutarate-dependent dioxygenases. The protein also contains two His motifs that are highly conserved in this family of dioxygenases. Thus, Fum3p is a 2-ketoglutarate-dependent dioxygenase required for the addition of the C-5 hydroxyl group of fumonisins.

Fumonisins are mycotoxins produced by the filamentous fungus *Fusarium verticillioides* (Sacc.) Nirenberg (synonym *F. moniliforme*, teleomorph *Gibberella moniliformis*, synonym *Gibberella fujikuroi* mating population A) (22, 25). The fungus is a widespread pathogen of maize. It causes ear and stalk rot and can colonize plant tissues without causing visible symptoms (4, 11). Fumonisins induce several animal diseases, including leukoencephalomalacia in horses (20, 23), pulmonary edema in swine (15), and cancer in rats (14). In some areas of China and South Africa, the consumption of fumonisin-contaminated maize is correlated with high incidences of human esophageal cancer (9, 34).

B-series fumonisins are typically the most abundant fumonisins in maize, with fumonisin B_1 (FB_1) making up to approximately 70% of the total content (25). Fumonisins consist of a linear 20-carbon backbone with hydroxyl, methyl, and tricarballylic acid moieties attached at various positions along the backbone (1). This structure is similar to sphinganine, an intermediate in the biosynthesis of sphingolipid. Fumonisins competitively inhibit sphinganine *N*-acyltransferase, thereby blocking the biosynthesis of sphingolipids, essential components of cell membranes (24, 30). This disruption may be the cause of the fumonisin-induced animal diseases.

The biosynthetic origins of fumonisins are partly established. Carbons 3 to 20 (C-3 to C-20) of the fumonisin backbone are derived from acetate and the amino group and C-1 and C-2 from alanine (2, 3). The methyl groups at C-12 and C-16 are derived from methionine (26), the C-3 hydroxyl group is from an acetate-derived carbonyl group, and the C-5 and C-10 hydroxyl groups are from molecular oxygen (7). The origin of vicinal diol at C-14 and C-15 is not totally clear, but it also could be from molecular oxygen (7). Recently, Proctor et al. (27–29, 32) cloned a 15-gene (*FUM1* and *FUM6* through *FUM19*) cluster responsible for the biosynthesis of fumonisins in *F. verticillioides*. The proteins deduced from the DNA sequence are consistent with the hypothesis that fumonisins are synthesized by a polyketide mechanism followed by modifications of the polyketide backbone. Among these genes, *FUM13* is known to encode a C-3 ketoreductase (6), and *FUM3* (previously called *FUM9*) is known to encode the C-5 hydroxylase (5). *FUM3* deletion mutants produce fumonisins that lack the C-5 hydroxyl group (i.e., FB_3 and FB_4), the same phenotype exhibited by mutants of *F. verticillioides* with defective alleles at the meiotically defined *Fum3* locus (12). So far, none of the *FUM* genes has been biochemically characterized. The data from *FUM3* mutants suggest that Fum3p catalyzes the C-5 hydroxylation of fumonisins, but direct evidence for the function of Fum3p is still lacking. Our objective in this study was to heterologously express *FUM3* and evaluate the Fum3p enzyme. We found that Fum3p is a 2-ketoglutarate-dependent dioxygenase required for C-5 hydroxylation of fumonisins.

MATERIALS AND METHODS

Materials, strains, and vectors. High-performance liquid chromatography (HPLC)-grade acetonitrile was from EM Science (Darmstadt, Germany) or from Fisher Scientific (Pittsburgh, Pa.). Yeast nitrogen base without amino acids (but with ammonium sulfate), uracil, raffinose, and salmon sperm DNA were from Sigma (St. Louis, Mo.). Standard FB_1, FB_2, FB_3, and FB_4 were gifts from Ronald D. Plattner (U.S. Department of Agriculture [USDA], Peoria, Ill.) or were purchased from Promec Unit, Medical Research Council, Tygerberg, South Africa. *F. verticillioides* wild-type strain A0149 (FGSC number 7600) was provided by David Gilchrist (University of California, Davis). Plasmid preparation and DNA extraction were carried out with Qiagen kits (Valencia, Calif.), and all other manipulations were carried out according to standard methods (31). *Escherichia coli* strain DH5α was used as the host for general DNA propagation, and cloning vectors were the pGEM-zf series from Promega (Madison, Wis.). *Saccharomyces cerevisiae* strain INVSc1 was purchased from Invitrogen (Carlsbad, Calif.). The strain is auxotrophic for histidine, leucine, tryptophan, and uracil. Yeast expression vectors pYES2/NT A, B, and C also were from Invitrogen.

Fum3p expression in yeast. The *FUM3* coding region was amplified by PCR with primers fum3-F-BamHI, 5′-CCGCATGGATCCAACAAGGAAAAGGTTCCC-3′ (underlined letters indicate BamHI site), and fum3-R-EcoRI, 5′-CCCGCGGAATTCTCAATCATCTAACCCAAG-3′ (underlined letters indicate

* Corresponding author. Mailing address: Chemistry Department, University of Nebraska—Lincoln, Lincoln, NE 68588-0304. Phone: (402) 472-2998. Fax: (402) 472-9402. E-mail: ldu@unlserve.unl.edu.

EcoRI site), with *F. verticillioides* genomic DNA as template and *Pfu*-Ultra polymerase (Stratagene, La Jolla, Calif.). The PCR product was digested with EcoRI and BamHI and ligated into pGEM-3zf (Promega) at the same sites to produce pDU1. After confirmation by DNA sequencing that no errors were introduced into the coding region during PCR, the cloned *FUM3* was transferred to pYES2/NT C via the BamHI and EcoRI sites to produce pDU2. The construct was subsequently transformed into yeast following the manufacturer's instructions (Invitrogen). For protein expression and extraction from the yeast cells, the same procedure was followed as described in the manufacturer's manual. The lysis buffer contained 50 mM sodium phosphate (pH 7.4), 5% glycerol, 1 mM phenylmethylsulfonyl fluoride. To precipitate proteins from the cell extracts, 0.338 g of ammonium sulfate was added slowly to 1 ml of extract solution (50% saturation at 4°C). After gentle shaking for 2 to 3 h, the solution was centrifuged at 16,000 × *g* at 4°C for 10 min. The supernatant was transferred to a new tube, and 0.201 g of ammonium sulfate was added to the solution (85% saturation at 4°C). The solution was gently shaken at 4°C overnight, and the precipitated proteins were collected by centrifugation at 16,000 × *g* at 4°C for 10 min. The protein pellets were redissolved in 500 μl of phosphate-buffered saline (PBS) buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4; pH 7.4) and dialyzed twice against 2,000 volumes of PBS buffer at 4°C. The proteins were loaded onto a Ni-nitrilotriacetic acid (NTA) column (Qiagen) preequilibrated with PBS buffer, and Fum3p was eluted in PBS buffer containing 250 mM imidazole. Finally, the eluents containing purified Fum3p were combined and desalted on a PD-10 column (Sephadex G-25; Pharmacia Biotech, Piscataway, N.J.) into 50 mM sodium phosphate buffer, pH 7.8, containing 1 mM dithiothreitol and 5% glycerol and stored at −80°C until used in in vitro assays.

Experiments in yeast cultures. One microliter of FB_3 stock solution (10 μg/μl, in 50% acetonitrile) was added to 200 μl of freshly prepared yeast cells, which had grown in induction medium at 30°C, 250 rpm, for 17 h. After the culture was incubated an additional 30 to 60 min under the same conditions, a 100-μl aliquot was centrifuged at 16,000 × *g* at room temperature for 8 min. The supernatant was transferred to a clean tube, and the cell pellet was combined with 20 μl of acetonitrile. The mixture was placed at −80°C for 2 to 3 h and thawed under running tap water. After this freezing-thawing procedure was repeated two more times, the mixture was centrifuged at 16,000 × *g* at room temperature for 8 min. The resulting supernatant was recovered and dried in a SpeedVac concentrator (Savant, Albertville, Minn.). The residue was suspended in 50 μl of H_2O to dissolve the trace amount of fumonisins. An aliquot (50 μl) of the solution was injected into an HPLC apparatus for analysis. As a control, a culture of INVSc1 carrying pYES2/NT C without *FUM3* was processed in the same manner.

Enzymatic assays for Fum3p. The enzyme assay followed previously described methods for 2-ketoglutarate-dependent dioxygenases (13, 19). A typical reaction mixture contained 10 μM Fum3p, 142 μM FB_3 or 290 μM FB_4, 100 μM freshly made $Fe(NH_4)_2(SO_4)_2$, 200 μM 2-ketoglutarate, 200 μM ascorbic acid, and 100 μg of catalase/μl in a total volume of 50 μl. After incubation at 30°C for 30 min, 50 μl of methanol was added to stop the reaction. The mixture was centrifuged at 16,000 × *g* at room temperature for 5 min, and a fraction of the supernatant (typically 50 μl) was injected into the HPLC apparatus for analysis. Reactions with boiled Fum3p (10 min in a boiled water bath), without 2-ketoglutarate, $Fe(NH_4)_2(SO_4)_2$, ascorbic acid, or catalase, also were conducted in parallel. Each reaction had at least three replications.

HPLC-ELSD and LC-ESMS methods. The HPLC system was a ProStar, model 210, from Varian (Walnut Creek, Calif.) with a column of Alltima C18LL, 5 μm, 250 by 4.6 mm inner diameter (Alltech, Deerfield, Ill.). The mobile phases were water-TFA (100:0.025 [vol/vol]) (A) and acetonitrile-TFA (100:0.025 [vol/vol]) (B), with a gradient of 0 to 40% B in A in the first 5 min, 40 to 60% B from 5 to 10 min, 60 to 80% B from 10 to 15 min, 80 to 80% B from 15 to 20 min, and 80 to 0% B from 20 to 25 min. The flow rate was 1.0 ml/min, and 50 μl was injected in analytic experiments. The conditions set for the evaporative laser scattering detector (ELSD 2000; Alltech) were 45°C of drift tube temperature, 2.0-liter/min nitrogen gas flow, and gain value of 1 in the impactor-on mode. For preparative HPLC, the respective fractions were collected directly from the column according to their retention times. A 50-μl aliquot of the concentrated fractions was reanalyzed by HPLC-ELSD to confirm the presence of the desired compounds. These salt-free fractions were used in liquid chromatography-electrospray mass spectrometry (LC-ESMS) analysis. LC-ESMS used a column of RFC_{18} Vydac, 5 μm, 250 by 1 mm inner diameter (C_{18} MassSpec; Vydac). The solvent system was the same as for HPLC-ELSD. The flow rate was 0.05 ml/min, and the injection volume was 50 μl. All positive electrospray spectra were acquired using a VG Platform II mass spectrometer.

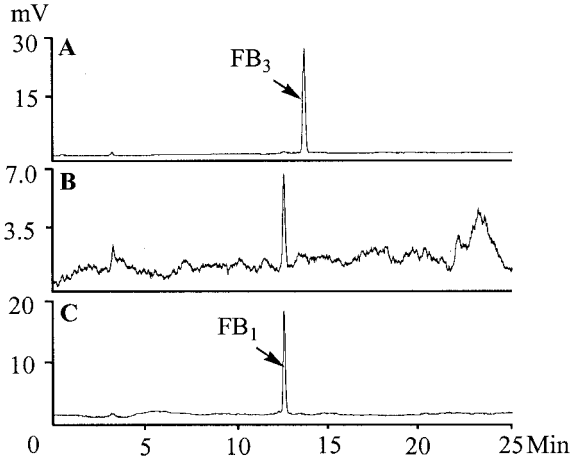

FIG. 1. HPLC-ELSD analysis of extracts from yeast cells fed FB_3. (A) Standard FB_3; (B) acetonitrile-water (1:1 [vol/vol]) extracts of yeast cells expressing *FUM3*; (C) standard FB_1.

RESULTS

Conversion of FB_3 to FB_1 by yeast cells expressing *FUM3*. Cultures of the yeast strain INVSc1 carrying pDU2 were fed FB_3. HPLC-ELSD analysis of extracts from these cultures revealed a peak with a retention time of 12.6 min that was not produced in control cultures (INVSc1 transformed with pYES2/NT) that had been fed FB_3. The retention time of this new peak was the same as that of FB_1 (Fig. 1). FB_3 had a retention time of 13.2 min under the same conditions. Since the only difference between FB_3 and FB_1 is a C-5 hydroxyl group, the results indicated that Fum3p expressed in yeast catalyzes C-5 hydroxylation of fumonisins.

Enzymatic activity of Fum3p. The Fum3p protein was expressed at a moderate level and was partially soluble under the experimental conditions used. Using a Ni-NTA affinity column, the His_6-tagged Fum3p was purified and yielded a 33-kDa band in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 2). Incubation of the purified protein with FB_3 in the presence of 2-ketoglutarate, iron(II), ascorbic acid, and catalase yielded a compound with the same retention time (12.3 min) as FB_1 in HPLC-ELSD (Fig. 3A). The fractions corresponding to all fumonisins (retention times from 12 to 15 min) on HPLC-ELSD were pooled and dried in vacuo. After redissolving in water, the fractions were analyzed by LC-ESMS. Two peaks appeared on the LC. The first had a retention time of 14.8 min and a molecular weight of 722.6, which are identical to the retention time and $[M+H]^+$ ion of the FB_1 standard (data not shown). The second peak had a retention time of 15.2 min and a molecular weight of 706.3, which are identical to the retention time and $[M+H]^+$ ion of the FB_3 standard (data not shown). The results showed that Fum3p catalyzes the conversion of FB_3 to FB_1. In a reaction mixture containing 5 μg (142 μM) of FB_3, the amount of FB_1 produced by Fum3p was 1,292 ± 91 ng, which represents a 24 to 28% conversion of FB_3 to FB_1. When boiled Fum3p was used in the reaction, no FB_1 was detected by HPLC-ELSD (Fig. 3B). A similar result was obtained when the reactions were run without iron(II) (Fig. 3C), 2-ketoglutarate (Fig. 3D), ascorbic acid

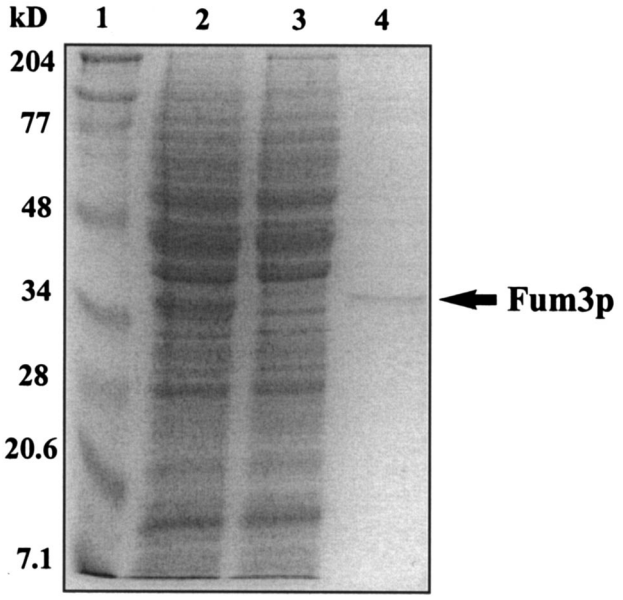

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel analysis of Fum3p expressed in *S. cerevisiae.* Lane 1, molecular mass markers; lane 2, total proteins after induction by isopropyl β-D-thiogalactopyranoside; lane 3, unbound proteins of Ni-NTA column; lane 4, 250 mM imidazole eluent of Ni-NTA column.

(data not shown), or catalase (data not shown). Thus, Fum3p has the characteristics of a typical 2-ketoglutarate-dependent dioxygenase.

Parallel to the reactions using FB_3 as substrate, a series of reactions with FB_4 as the substrate also were conducted. Under the same conditions, the standard FB_4 had a retention time of 14.4 min, and the standard FB_2 had a retention time of 13.4 min on HPLC-ELSD (data not shown). However, no observable FB_2 was produced in any of the Fum3p reaction mixtures with FB_4 as the substrate. Varying the concentration of Fum3p or FB_4, with or without the cosubstrate or cofactors, in the reactions did not change these results.

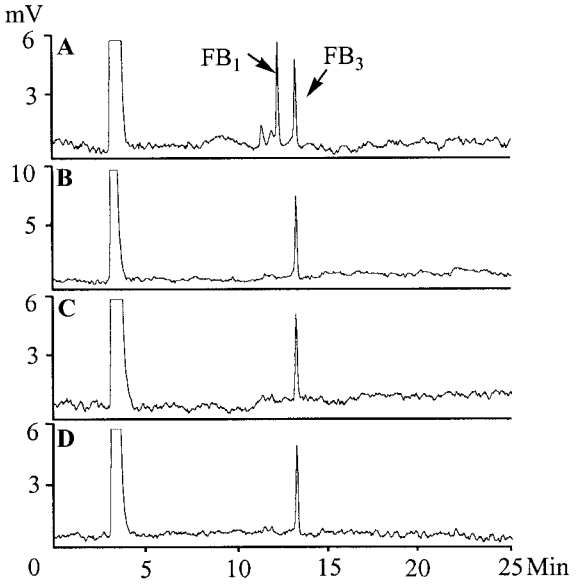

FIG. 3. HPLC-ELSD analysis of Fum3p reactions using FB_3 as substrate. (A) Full reaction mixture containing 10 μM Fum3p, 142 μM FB_3, 100 μM $Fe(NH_4)_2(SO_4)_2$, 200 μM 2-ketoglutarate, 200 μM ascorbic acid, and 100 μg of catalase/μl in a total volume of 50 μl. (B) Full reaction mixture but with boiled Fum3p. (C) Full reaction without $Fe(NH_4)_2(SO_4)_2$. (D) Full reaction mixture without 2-ketoglutarate.

DISCUSSION

So far, eight (*FUM1*, *FUM3*, *FUM6*, *FUM8*, *FUM13*, *FUM17*, *FUM18*, and *FUM19*) of the 15 *FUM* genes have been disrupted or deleted by homologous recombination (5, 6, 27, 28, 32), but only two (*FUM3* and *FUM13*) of these disruptions resulted in the accumulation of detectable intermediates (5, 6). *FUM13* encodes a ketoreductase required for the reduction of the C-3 keto group of the carbon backbone (6), and *FUM3* encodes a hydroxylase required for the hydroxylation of the C-5 position (5). We biochemically confirmed that the function of the *FUM3*-encoded protein was to hydroxylate the C-5 position of fumonisins. This conclusion is based on both in vivo and in vitro conversion of FB_3 to FB_1 by the heterologously expressed Fum3p enzyme.

FUM3 is a 903-bp gene located in the middle of the *FUM* gene cluster (27, 32). Fum3p shares 30 to 40% identity at the amino acid level to several hypothetical proteins, including a hypothetical protein (AAK01519) from *Pseudomonas aeruginosa*, a conserved hypothetical protein (AAN68422) from *Pseudomonas putida* KT2440, and hypothetical protein Rv3633 (NP_218150) from *Mycobacterium tuberculosis* H37Rv, but it did not exhibit any significant similarity to proteins with known functions. In several short regions (50 to 60 amino acid residues), Fum3p has some similarity (approximately 40%) to epoxidase subunit A from *Penicillium decumbens* (BAA75924) (35), a probable phytanoyl-coenzyme A 2-hydroxylase from *Homo sapiens* (NP_006205) (8), *Rattus norvegicus* (P57093) (18), and *Mus musculus* (NP_034856) (17), L-proline 4-hydroxylase from *Dactylosporangium* sp. (BAA20094) (33), and MmcH (a putative 2-ketoglutarate-dependent dioxygenase) from *Streptomyces lavendulae* (AF127374) (21). Thus, the biochemical characterization of Fum3p has implications for the function of the hypothetical proteins identified in other organisms.

Fum3p belongs to the family of 2-ketoglutarate-dependent dioxygenases. These enzymes catalyze a wide range of substrate conversions, including hydroxylation, desaturation, and epoxidation, using O_2 and 2-ketoglutarate as cosubstrates and Fe^{2+} as a cofactor (10, 16). One atom of O_2 is inserted into 2-ketoglutarate, which is subsequently decomposed to give succinate and CO_2, and the other is inserted in the second organic product (e.g., FB_1). Most of these oxygenases require, but not absolutely, ascorbate and catalase for optimal substrate conversion in vitro (10, 16). The role of ascorbate probably is indirect and unrelated to the reaction mechanism, whereas the role of catalase is presumably to protect the dioxygenase from the H_2O_2 generated in the reaction medium (10, 16). The activity of Fum3p was clearly dependent on 2-ketoglutarate, Fe^{2+}, ascorbate, and catalase. The conclusion that Fum3p is a 2-ketoglutarate-dependent dioxygenase also is supported by a more detailed sequence analysis. Although an initial database

search yielded no clear matches, a careful analysis of the deduced amino acid sequence of the *FUM3* product identified His-1 and His-2 motifs, which are highly conserved in this group of dioxygenases (13, 19, 33). The two motifs usually are arranged in the general pattern of H-X-D-X_{53-57}-H (X being any amino acid residue), in which the two histidines and one aspartate constitute the endogenous ligands of the Fe^{2+} active site. Thus, the residues H_{146}, D_{148}, and H_{221} of Fum3p could be the facial triad for its active site common to all 2-ketoglutarate-dependent dioxygenases.

Fum3p was expected to convert FB_4 to FB_2. Like FB_1, FB_2 contains the C-5 hydroxyl group, but not the C-10 hydroxyl. FB_3 and FB_4 have the same structure except that FB_3 has the C-10 hydroxyl group. The failure of Fum3p to convert FB_4 to FB_2 suggests that the presence of a C-10 hydroxyl group may be important for the C-5 hydroxylation activity of Fum3p. The enzyme responsible for the addition of the C-5 hydroxyl group of FB_2 has not been identified. One possibility is that FB_4 may not be the direct precursor of FB_2. Further biochemical characterization of other redox enzymes in the *FUM* cluster, e.g., *FUM6, FUM12*, *FUM 13*, and *FUM15*, should shed further light on the origins of the hydroxyl groups of fumonisins.

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