Acetyltransferase activity in Pseudomonas sp. capable of acetyling the C-4 hydroxy group of nivalenol-type trichothecenes

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Key Words: acetylase; Fusarium; mycotoxin; Pseudomonas; triacetylated trichothecene

Abbreviations: 15-ANIV, 15-acetylnivalenol; 3-ANIV, 3-acetylnivalenol; 3,15-diANIV, 3,15-diacetylnivalenol; 3,4,15-triANIV, 3,4,15-triacetylnivalenol; DON, deoxynivalenol; NIV, nivalenol

Trichothecenes are sesquiterpene mycotoxins, with a common tricyclic 12,13-epoxytrichothec-9-ene skeleton, produced by fungi such as Fusarium, Trichothecium, and Myrothecium, and they inhibit protein synthesis through binding to the ribosomal peptidyltransferase site on the 60S subunit in eukaryotic cells (Kimura et al., 2007). Naturally-occurring trichothecenes, such as T-2 toxin produced by F. sporotrichioides and deoxynivalenol (DON) and nivalenol (NIV) produced by F. graminearum, are found in important crops, such as wheat and corn, across the world (Desjardins, 2009; Yazar and Omurtag, 2008). Animals and humans exposed to these trichothecenes develop symptoms, such as diarrhea, vomiting, and alimentary toxic aleukia. Hence, trichothecenes represent a major challenge for food and feed safety (Marin et al., 2013).

In order to control trichothecene mycotoxins, it is essential to monitor contamination levels in food and feed samples, for which enzyme-linked immunosorbent assay (ELISA) is an easy and convenient method. At present, ELISA is commercially available for DON and T-2 toxin, but not for NIV, since no primary antibodies with sufficient affinity for NIV have been produced so far. Instead, there exist antibodies against 3,4,15-triacetylnivalenol (3,4,15-triANIV), thus, acetylation of NIV to 3,4,15- triANIV, would make the immunoassay possible (Wang and Chu, 1991; Yoshizawa et al., 2004). For this purpose, trichothecene biosynthetic (TRI) enzymes seem to provide a promising and attractive tool that avoids tedious organic synthesis. Although recombinant trichothecene 3-O-acetylase (TRI101) (Kimura et al., 1998) and 15-O-acetylase (TRI3) (McCormick et al., 1996; Tokai et al., 2008) are available, no enzymes have yet been developed for 4-O-acetylation of the trichothecene skeleton. Tri7 has been shown to be involved in the C-4 acetylation step, based on results from targeted gene disruption experiments (Brown et al., 2001; Lee et al., 2002). However, attempts to express Tri7 in other host microorganisms, such as Escherichia coli, Saccharomyces cerevisiae, and Aspergillus oryzae, have been unsuccessful (our unpublished results and the results of other groups published in “Progress Reports on Integrated Research Program for Functionality and Safety of Food Toward an Establishment of Healthy Diet”, Forestry and Fisheries Research Council Secretariat (January 2013; http://agriknowledge.affrc.go.jp/RN/2039017283.pdf)).

Given reports of soil microorganisms capable of metabolizing trichothecenes (Islam et al., 2012; Sato et al., 2012; Shima et al., 1997; Ueno et al., 1983), screening for those...
Microbial acetylation of nivalenol with trichothecene 4-\textit{O}-acetylase activity may accelerate the discovery of a replacement for the currently unavailable TRI7p. In the present study, we have performed a large-scale screening for soil microorganisms with trichothecene 4-\textit{O}-acetylase activities, and measured substrate specificities of NIV and its closely related derivatives.

In order to prepare commercially unavailable rare substrates of C-4 acetylase, such as 3,15-diacetylnivalenol (3,15-diANIV), 15-acetylnivalenol (15-ANIV), and 3-acetyldeoxynivalenol (3-ADON)/DON chemotype of \textit{F. graminearum} strain F15 transformed with NIV-type \textit{FgTri13} produced only a limited amount of trichothecene 3-ANIV (Maeda et al., 2012), we designed an ectopic expression vector with a strong \textit{Aspergillus nidulans} TEF1\textit{a} promoter (Nakajima et al., 2014). The vector (Fig. S3) was transformed into the 3-ADON/DON chemotype of \textit{F. graminearum} strain F15, and transformants producing a relatively fair amount of rare trichothecene 3-ANIV were obtained (see Fig. S4).

In order to identify the strain No. 3010, genomic DNA was obtained by a standard phenol/chloroform extraction. The 16S rRNA gene was selectively amplified by PCR using forward and reverse primers 27F and 1492R (Table S1). PCR products were purified and treated with exonuclease I (TaKaRa Bio Inc., Shiga, Japan) and shrimp alkaline phosphatase (Promega Corp., Fitchburg, WI, USA). Purified PCR products were sequenced using the ABI PRISM BigDye Terminator v3.1 cycle sequencing ready reaction kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) on the ABI PRISM 310 Genetic Analyzer, follow-
ing the manufacturer’s protocol (Thermo Fisher Scientific Inc.). Primers 351R, 531F, 692R, 811F, 953R, and 1182F were used (Table S1). The same complete sequence (1471 bp, accession No. LC128308) of 16S rRNA gene of the strain No. 3010 was determined and NCBI BLAST search was performed. The sequence similarity was found with Pseudomonas fluorescens strain HZN1 (99.8%, GU358073) and Pseudomonas vancouverensis strain DaH-51T (99.8%, NR_041953), and some morphological and physiological characteristics indicated that the strain No. 3010 belongs to the genus Pseudomonas (Fig. S5).

The strain No. 3010, was inoculated in 100 mL of TSB medium in a 500-mL flask, and incubated at 30°C at 150 rpm for 3 days. A cell culture aliquot (80 mL) was centrifuged at 8,000 × g for 15 min, following which 4 mL of sample buffer (10 mM sodium phosphate buffer pH 7.0, 5 mM EDTA, 1 mM PMSF) was added to the pellet and sonicated (TOMY Seiko Co. Ltd. Tokyo, Japan). The sonicate was centrifuged at 12,000 × g for 15 min, filtered through a 0.2-µm pore filter, and the resulting filtrate was used as crude enzyme.

The enzymatic reaction was carried out as follows: 10 µL of 1 M each buffer (sodium phosphate buffer in Figs. 1, 2, 3, S6, S7; MES buffer in Fig. 1; sodium acetate buffer in Fig. 1), 1 µL of 100 mM acetyl CoA, and 5 µg of substrate were added to the crude enzyme (1.0 mg protein) in a total volume of 100 µL. In this case, the molar ratio of acetyl CoA to trichothecene was greater than 6.

The reaction was terminated and trichothecenes were extracted by the addition of 100 µL of ethyl acetate, or 100 µL of acetonitrile supplemented with 20 µL of 5 M NaCl. This process was repeated four times. The extract was dried under nitrogen, the residue was resolved in 200 µL of 50% ethanol and analyzed by HPLC (LC-2000 plus; JASCO Corp., Tokyo, Japan) using a C18 reverse phase column (4.60 × 250 mm, PEGASIL ODS SP100; Senshu Scientific Co., Ltd., Tokyo, Japan). Trichothecenes were eluted using the mobile phase of acetonitrile and water, according to the following steps: 5% acetonitrile for 5 min, a linear gradient of 5% to 20% acetonitrile for 12 min, 20% to 50% acetonitrile for 9 min, 50% acetonitrile held for 6 min, then 5% acetonitrile held for 8 min. The eluates were detected by measuring the absorbance at 254 nm and the molar concentration of each trichothecene was calculated from the peak area.

The enzymatic properties of C-4 acetylase were examined using 3,15-diANIV as a substrate. The optimal pH of the crude enzyme was 6.3, and enzyme activity was higher in a phosphate buffer than in an acetate buffer (Fig. 1). In order to determine enzyme stability under high or low pH conditions, the pH of the crude enzyme solution was adjusted to 4.3 and 6.3 using 100 mM acetate buffer, or 9.2 using 100 mM carbonate buffer for 1 h, followed by dialysis against 10 mM phosphate buffer (pH 7.1). Activity of the crude enzyme was almost identical at all tested pH values, confirming that the enzyme was stable between pH 4.3 and 9.2 (data not shown).

The reaction rate of the enzyme was highest at 40°C (Fig. 2), but the activity disappeared upon heating to 60°C for 10 min (data not shown). About half of the enzymatic activity was sustained after 5 days pre-incubation at 30°C (Fig. S6), indicating that the enzyme was unstable at 60°C, but fairly stable at 30°C for several days.

Analysis of substrate specificity was also performed. NIV, 3-ANIV, 15-ANIV or 3,15-diANIV (5 µg) were used as substrates, and reactions were carried out at pH 6.3 in 100 mM phosphate buffer. The enzyme showed the highest activity toward the C-4 hydroxyl group of 3,15-diANIV and, to a lesser extent, of 3-ANIV, but no acetylation was observed with NIV and 15-ANIV (Fig. 3). In the biosynthesis of trichothecenes, isotrichodermol, the first intermediate with a toxic 12,13-epoxytrichothec-9-ene skeleton, is first acetylated at C-3 and subsequent hydroxylations, acetylations, and oxidation proceed with a 3-O-acetyl group attached to the trichothecene skeleton. Also, C-4 acetylation by TRI7p occurs only after the formation of 3,15-diANIV (Kimura et al., 2007). From this set of events, it is assumed that the 3-O-acetyl group is required for 4-O-acetylase activity by TRI7p. In this regard, it is noteworthy to mention that the microbial C-4 acetylase showed a biased preference for 3,15-diANIV, the authentic biosynthetic intermediate, and less preference for 3-ANIV, an unnatural trichothecene, both of which are acetylated at C-3. It is possible that the C-3 acetyl sterically shields the highly reactive 12,13-epoxy group, allowing other biosynthetic enzymes to easily accommodate their substrates in the active center. Neither C-3 nor C-15 hydroxyl groups were acetylated by this microbial enzyme. The kinetic parameter Km for 3,15-diANIV was found to be 107 M at 40°C in 100 mM phosphate buffer (pH 6.3) (Fig. S7).

In order to control trichothecenes, an easy and efficient detection system for NIV is urgently required. In contrast to DON and T-2 toxin, no appropriate antibodies are available for NIV detection by ELISA, and no immunoassays have been developed. However, since TRI101 and TRI3 conveniently exhibit optimal activities around a neutral pH, the combined usage of these TRI enzymes and the C-4 acetylase from the strain No. 3010 will contribute to acetylate NIV-type trichothecenes into 3,4,15-triANIV, for which antibodies are already available (Yoshizawa et al., 2004). Thus, collective detection of NIV-type trichothecenes using ELISA might become possible in the near future. This study reports on the potential use of NIV mycotoxin detection for the assessment and maintenance of food safety.

Acknowledgments

This work was supported by JSPS KAKENHI Grant Number 15K07459, the Inoue Enryo Memorial Research Fund, and the Iijima Memorial Foundation for the Promotion of Food Science and Technology.

Supplementary Materials

Supplementary figures and table are available in our J-STAGE site (http://www.jstage.jst.go.jp/browse/jgam).

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