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Involvement of two cytosolic enzymes and a novel intermediate, 5'-oxoaverantin, in the pathway from 5'-hydroxyaverantin to averufin in aflatoxin biosynthesis

Emi SAKUNO [*1, 2], Hiromitsu NAKAJIMA [*1], and Kimiko YABE [*2]

[*1] Faculty of Agriculture, Tottori University
(Koyama, Tottori 680-8553, Japan)
[*2] National Food Research Institute
(Tsukuba, Ibaraki 305-8642, Japan)

Summary

In aflatoxin biosynthesis 5'-hydroxyaverantin (HAVN) is converted to averufin (AVR). We demonstrated that this step is composed of two enzymatic steps: an enzyme, HAVN dehydrogenase, catalyzes the reaction from HAVN to a new intermediate and another new enzyme catalyzes the following reaction from the intermediate to AVR. The intermediate was assigned to be 5'-oxoaverantin (OAVN) by spectroscopic methods. We purified HAVN dehydrogenase and OAVN cyclase from the cytosol fraction of *A. parasiticus* and characterized. MALDI-TOFMS analysis of tryptic peptides of the purified HAVN dehydrogenase revealed that this enzyme is encoded by *adhA* gene in the aflatoxin gene cluster of *A. parasiticus*.

Key words : aflatoxin, biosynthesis, 5'-hydroxyaverantin, averufin, enzyme

Aflatoxins comprise a group of polyketide-derived secondary metabolites mainly produced by certain strains of *Aspergillus flavus* and *A. parasiticus*. They are toxic, mutagenic, and carcinogenic to a variety of animal species and humans: their contamination of agricultural commodities has serious deleterious effects on health of animals and humans [1, 2]. In the early step of aflatoxin biosynthesis, 5'-hydroxyaverantin (HAVN) is converted to averufin (AVR). Chang et al. recently reported that disruption of the *adhA* gene in the aflatoxin gene cluster of *A. parasiticus* caused accumulation of HAVN, indicating that this gene might encode HAVN dehydrogenase [3]. In this study we purified HAVN dehydrogenase from the cytosol fraction of *A. parasiticus* NIAH-26 [4-6] and demonstrated that two enzymes are involved in the pathway from HAVN to AVR.

The desalted cytosol fraction of *A. parasiticus* NIAH-26 was applied onto a Mono Q column. When HAVN was incubated with each fraction, the fractions from no. 26 to 36 produced a new substance which was neither HAVN nor AVR (Fig. 1A). In contrast

the fractions from no. 38 to 42 yielded larger amounts of AVR than did the other fractions. These results suggested that the cytosol fraction contains two different enzymes, which catalyze the formation of the unknown substance and of AVR, respectively. In order to confirm this the following two-step procedure was devised. HAVN was first incubated with pooled fractions 29 to 33 to produce unknown substance, and then each fraction obtained after Mono Q column chromatography was added to an aliquot of the resultant reaction mixture. AVR was produced significantly when each fraction from no. 38 to 54 was added, whereas other fractions did not show any change (Fig. 1B). These results indicated that fractions 26 to 36 contained HAVN dehydrogenase which catalyzed the conversion of HAVN to the new intermediate and that fractions 38 to 54 contained another enzyme which catalyzed conversion of the intermediate to AVR.

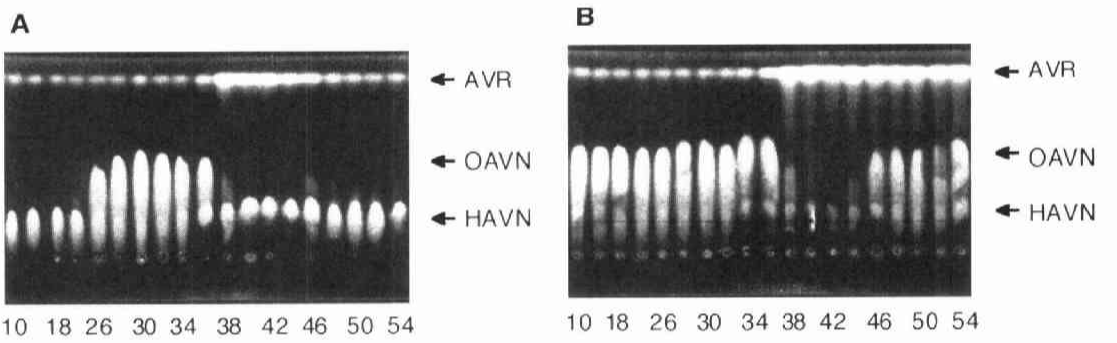

Fig. 1. Mono Q ion-exchange chromatography of cytosol fraction from *A. parasiticus* NIAH-26.

A: Each fraction of Mono Q ion-exchange chromatography was incubated with HAVN in the presence of NAD. B: HAVN was first changed to OAVN by incubation of HAVN with the fractions containing HAVN dehydrogenase (No. 29-33 fractions, See A). Each fraction of the Mono Q chromatography was then added to an aliquot of the first reaction mixture and incubated. The reaction products were analyzed by TLC.

We succeeded in isolating the new intermediate from the preparation obtained after incubating HAVN with the purified HAVN dehydrogenase. The structure was assigned to be OAVN (Fig. 2) by comparing the spectroscopic data of the new intermediate to those of HAVN.

HAVN dehydrogenase was purified from the cytosol fraction of *A. parasiticus* NIAH-26 [4, 5] after ammonium sulfate fractionation and the successive chromatographic steps. The highly active fractions 29 to 37 obtained by the final Mono Q chromatography step showed four bands on SDS-PAGE analysis. A protein band (28 kDa) was correlated with the enzyme activity. The HAVN dehydrogenase requires NAD, but not NADP, as a cofactor. Based on gel filtration chromatography, the native molecular mass of HAVN

dehydrogenase was estimated to be 60 kDa. The optimum pH of the enzyme activity was between 7.5 and 8.5. The optimum temperature was around 30 to 35 ℃. The values of K_m and V_{max} of the enzyme for HAVN were estimated to be 83 μM and 2.3 μmol mg of protein^{-1} min^{-1}, respectively. Although we tried to determine the N-terminal sequence of the enzyme, no sequence could be detected, indicating that the N-terminus of this enzyme may be blocked. Thus the purified HAVN dehydrogenase was digested by trypsin and the products were analyzed by MALDI-TOFMS. Seven peptide fragments were observed, and the molecular masses matched the theoretical fragment masses from the gene product of *adhA* which was reported to be the gene encoding HAVN dehydrogenase[3]. This result demonstrated that HAVN dehydrogenase was encoded by the *adhA* gene. Furthermore, the fragment corresponding to the N-terminal 16 amino acids at a molecular mass of 1777.9 indicated that the N-terminal methionine of HAVN dehydrogenase is posttranslationally acetylated and oxidized to form acetyl methionine

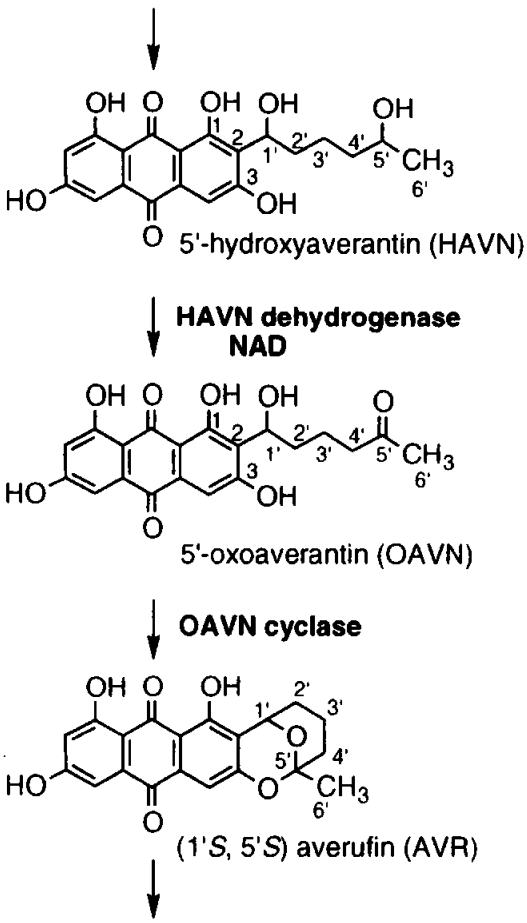

Fig. 2. Pathway from HAVN to AVR clarified in this study.
HAVN is converted to OAVN by HAVN dehydrogenase which requires NAD, but not NADP, for its activity. OAVN is then converted to AVR by OAVN cyclase.

sulfoxide. Methionines at either or both of the 172nd and 179th positions of the amino acid sequence are also oxidized in some, but not all, enzyme molecules.

OAVN cyclase was purified from the cytosol fraction of *A. parasiticus* NIAH-26 after ammonium sulfate fractionation and the successive chromatographic steps. After the final Resource PHE chromatography the peak fraction of the enzyme activity showed three protein bands, one major band of 79 kDa and two minor bands by SDS-PAGE. The 79 kDa protein correlated with the enzyme activity in the last three purification steps, indicating that this protein was OAVN cyclase. The OAVN cyclase is a homodimer composed of 79 kDa subunits, and neither NADP(H) nor NAD(H) was required for the enzyme activity. The optimum pH was between 6 and 7. The optimum temperature was broad, from 25 to 45 ℃. The kinetic values K_m and V_{max} of the enzyme for OAVN were estimated to be 20 μM and 6.67 μmol mg of protein^{-1} min^{-1}, respectively.

In this study we demonstrated that the pathway of HAVN→OAVN→AVR is included in aflatoxin biosynthesis (Fig. 2). We previously proposed that OAVN would be produced as a transient intermediate between HAVN and AVR and that OAVN would cyclize to form AVR spontaneously, not enzymatically. Since OAVN is unstable enough to form AVR easily and OAVN cyclase does not require any cofactors, the reaction from HAVN to AVR had appeared to be a one-enzyme reaction depending on the presence of NAD[4, 6]. In this work we unequivocally demonstrated the involvement of an enzyme in the reaction from OAVN to AVR.

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