Biosci. Biotechnol. Biochem., **69** (11), 2254–2257, 2005

Note

Purification and Characterization of Aldoxime Dehydratase of the Head Blight Fungus, *Fusarium graminearum*

Yasuo KATO and Yasuhisa ASANO†

Biotechnology Research Center, Faculty of Engineering, Toyama Prefectural University, 5180 Kurokawa, Toyama 939-0398, Japan

Received June 29, 2005; Accepted July 9, 2005

Fungal aldoxime dehydratase (Oxd) of *Fusarium graminearum* MAFF305135 was purified and characterized for the first time from its overexpressing *Escherichia coli* transformant. The enzyme showed about 20% identity with known Oxds, and had similar enzymatic properties with nitrilase-linked Oxd from the *Bacillus* strain. It belongs to a group of phenylacetaldoxime dehydratases (EC 4.99.1.7), based on its substrate specificity and kinetic analysis.

Key words: aldoxime dehydratase; aldoxime-nitrile pathway; nitrilase; *Fusarium graminearum*; characterization

We have been studying microbial aldoxime metabolism and have found from gene sequences and enzymatic evidence that aldoxime is metabolized into the corresponding carboxylic acids through nitrile by a combination of a novel enzyme, aldoxime dehydratase (Oxd; EC 4.99.1.-), and nitrile-hydrolyzing enzymes such as nitrilase (Nit), and nitrile hydratase (NHase) and/or amidase. The pathway involving the enzymes was named the "aldoxime-nitrile pathway".[1–10] Among these enzymes, we found that Oxd is unique because it contains heme as a prosthetic group despite its simple catalytic activity, involving a dehydration reaction.[3,6,7,9–11] Some reaction mechanisms of Oxds have been elucidated,[10–13] but their details are still unknown. In order to obtain and compare biochemical properties of Oxds from various sources, it is important to identify characteristics of Oxd from eukaryotes, since Oxds have been purified and characterized only from bacterial sources such as *Bacillus*, *Pseudomonas*, and *Rhodococcus*.[3,6,7,9,11] It has been reported that strains belonging to the genus *Fusarium* are effective producers of Nit.[14–16] Nit was used as a potential catalyst to produce monocarboxylic acid from di- or tri-nitriles.[14,15] We have clarified that the *Fusarium* strain has both Oxd and Nit activities,[2] but no enzymatic studies including the primary structure on *Fusarium* Oxd have been carried out. We searched genome databases and found that *Fusarium graminearum* (telemorph *Gibberella zeae*), which causes *Fusarium* head blight of wheat and barley, contains a gene encoding an Oxd homolog (protein code: 78267.1) at location 165270–166361 on chromosome 4 of that strain [accession no. AACM01000259]. The present work reports cloning and overexpression of this gene in *E. coli*. The protein encoded by the gene was purified in a His_6-tagged form and subsequently characterized. This is the first report on purification and characterization of Oxd from eukaryotes.

F. graminearum Schwabe MAFF (gene bank of the Ministry of Agriculture, Foresty, and Fisheries of Japan) 305135 was used as DNA source since the original genome-sequenced strain *F. graminearum* PH-1 could not be obtained. The strain was cultured on a potato-dextrose agar medium as described previously.[2] The putative Oxd was originally annotated as a hypothetical protein, but we found remarkable (20–21%) sequence identities of the protein with known bacterial Oxds, as shown in Fig. 1. To obtain the complete coding region for the gene, PCR-amplification was performed. Since the genome database of *F. graminearum* suggests that the Oxd homolog gene does not contain introns, we amplified the gene directly from the genome of the strain with the following primers designed from its open reading frame: OxdFG-F (5′-TAAGGATCCGCTTCG-GTCACGTTTCCC-3′) contained an engineered *Bam*H1 site (underlined) and 5′-region of the gene (ATG start codon was deleted), while OxdFG-R (5′-CTACCATGG-*CTA*CCACTCTATCGGCTT-3′) had an engineered *Nco*I site (underlined) and 3′-region of the gene including stop codon (italics). The vector used for expression, pUC18-His, was modified pUC18, such that a His_6-tag and short polypeptides derived from its multicloning sites (MHHHHHHGMASMTGGQQMGRDP) were placed at N-terminal Met position of the Oxd homolog. PCR was performed in reactions (30 µl) containing 0.1–5 µg chromosomal DNA of *F. graminearum* extracted as described previously,[17] 30 pmol each of the primers, 300 µM dNTPs, 1 × PCR buffer, and 2.5 units of Blend-*Taq* polymerase (Toyobo, Osaka,

† To whom correspondence should be addressed. Tel: +81-766-56-7500; Fax: +81-766-56-2498; E-mail: asano@pu-toyama.ac.jp
Abbreviations: Oxd, aldoxime dehydratase; PAOx, phenylacetaldoxime; IAOx, indoleacetaldoxime

Fig. 1. Amino Acid Sequence Comparison of Oxds from *Rhodococcus* sp. N-771 (OxdRE), *R. globerulus* A-4 (OxdRG), *Pseudomonas* sp. K-9 (OxdK), *P. chlororaphis* B23 (OxdA), *Bacillus* sp. OxB-1 (OxdB), and *F. graminearum* MAFF305135 (OxdFG, this study).

Identical and similar amino acids are indicated by asterisks and colons respectively. Conserved distal and proximal histidine residues are boxed by solid and dotted lines respectively.

Table 1. Comparison of the Properties of Oxds from *Bacillus* sp. OxB-1 (OxdB), *R. erythropolis* N-771 (OxdRE), *R. globerulus* A-4 (OxdRG), *P. chlororaphis* B23 (OxdA), *Pseudomonas* sp. K-9 (OxdK), and *F. graminearum* MAFF305135 (OxdFG, this study)

Properties	OxdB[a]	OxdRE[a]	OxdRG[a]	OxdA	OxdK[a]	OxdFG[a]
Molecular weight (M_r)						
Native	42,000	80,000	80,000	76,400	85,000	34,100
Sequence	40,972	44,794	44,817	40,127	44,511	44,070
Number of subunits	1	2	2	2	2	1
Soret peak (nm) (ferric form)	407	409	409	408	408	420
(ferrous form)	432	428	428	428	428	431
Specific activity[b]	851	562	633	197[c]	2.25	24.8
Optimum pH[d]	7.0	8.0	8.0	5.5[c]	7.0	5.5
Temp. (°C)[e]	30	30	30	45[c]	20	25
Stability pH[d]	6.5–8.0	6.0–9.5	6.0–9.5	6.0–8.0	5.5–6.5	4.5–8.0
Temp. (°C)[e]	<45	<40	<40	<40	<30	<20

[a]As His_6-tagged form at the N-terminus.
[b]Enzyme activity for *Z*-PAOx (U/mg) was measured under anaerobic conditions.
[c]*n*-Butyraldoxime was used as a substrate.
[d]The effects of pH on enzyme activity and stability were investigated in several 0.1 M buffers at various pHs using *Z*-PAOx as the substrate.
[e]The effects of temperature on enzyme activity and stability were investigated at various temperatures between 20 °C and 80 °C in 0.1 M KPB (pH 7.0).

Japan) according to a previously described method.[8] The resulting amplified band (1 kbp) was extracted from an agarose gel, digested with *Bam*H1 and *Nco*I, and ligated with pUC18-His vector to obtain pOxdFG for further sequencing and overexpression. We sequenced PCR-amplified fragments, and found 35 differences on the nucleic acid sequence from that found in the genome database: that caused 10 amino acid replacements and 25 silent mutations. These results might be due to differences between the strains used for DNA isolation and genome sequencing. The nucleotide sequence of the gene identified in this study was deposited in DDBJ/EMBL/GenBank databases under accession no. AB214653.

The protein was expressed in *E. coli* JM109 cells harboring pOxdFG in LB medium, and aldoxime dehydration activity was measured as described for the other Oxds.[3–10] Cell-free extract of the transformant exhibited stoichiometric dehydration of *Z*-phenylacetaldoxime (*Z*-PAOx) into phenylacetonitrile at pH 7.0. It was clear that the gene (*oxd*) coding for Oxd had a new primary structure, and we tentatively named the protein OxdFG. The transformant was grown under various culture conditions (medium volume, temperature, medium, *etc*.) as we previously examined for Oxds of *Bacillus* sp. OxB-1 (OxdB),[5] *R. erythropolis* N-771 (OxdRE),[7] and *Pseudomonas* sp. K-9 (OxdK),[9] and *Z*-PAOx dehydration activity was measured. The highest activity was seen when the strain was grown at about 30 °C in a high medium volume of Terrific medium,[18] as we had seen when expressing other Oxds.[5,7,8] It reached about 4,000 U/l culture.

OxdFG was purified to homogeneity from the *E. coli* strain in its N-His_6-tagged form using a Co-chelated Talon affinity column (BD Bioscience, Palo Alto, CA), as previously described for Oxds,[9,10] and was characterized. Table 1 summarizes the properties of OxdFG, and those of Oxds from other sources. Purified OxdFG showed a single band on SDS–PAGE, in agreement with its M_r deduced from the gene sequence. The native M_r of

Table 2. Kinetic Parameters of N-His_6-Tagged OxdFG from *F. graminearum* MAFF305135

Aldoxime	K_m (mM)	V_{max} (U/mg)	V_{max}/K_m (U/mg/mM)	Relative activity (%)[a]
Arylalkylaldoxime				
Z-Phenylacetaldoxime	3.52	28.2	8.01	100
E/*Z*-2-Phenylpropionaldoxime	3.71	18.1	4.88	68.6
Z-3-Phenylpropionaldoxime	2.76	20.4	7.39	104
E/*Z*-Indoleacetaldoxime	1.46	19.3	13.2	125
E/*Z*-Mandelaldoxime	1.70	2.32	1.36	16.1
E/*Z*-4-Phenybutyraldoxime	1.79	14.1	7.88	79.2
Alkylaldoxime				
E/*Z*-*n*-Butyraldoxime	2.87	20.4	7.11	107
E/*Z*-*n*-Valeraldoxime	10.1	88.8	8.79	341
E/*Z*-Isovaleraldoxime	2.66	23.1	8.68	92.7
E/*Z*-*n*-Capronaldoxime	0.802	3.60	4.48	17.6

[a]Measured at 5 mM concentration. The enzyme activity for *Z*-PAOx dehydration activity was taken to be 100%. The relative activities for the other aldoximes are as follows: *Z*-cinnamaldehyde oxime (17.9%), *E*/*Z*-propionaldoxime (10.6%), *E*/*Z*-isobutyraldoxime (5.97%), and *E*/*Z*-cyclohexanecarboxaldehyde oxime (11.6%).

the enzyme was estimated to be about 31,400, according to the results of gel filtration chromatography with Superdex200 (Amersham, Uppsala, Sweden), indicating that the enzyme existed in a monomeric form, like OxdB.[3)] The enzyme showed an orange color and had heme *b* as a prosthetic group, as seen by heme-extraction and analysis, as described previously.[3,6,7,9)] The absorption maximum (Soret peak) of the purified OxdFG was at 420 nm, which was shifted to 431 nm by reduction with $Na_2S_2O_4$. The Soret peak of the reduced enzyme was further shifted to 424 nm by bubbling CO gas. These results suggest that the enzyme was purified in a ferric form, but its absorption spectrum was different from those of Oxds from various sources, whose Soret peak of ferric form is about 407–408 nm.[6,7,9,10)] The differences in Soret peak between OxdFG and other Oxds presumably derive from diversity in the structure of Oxds, although the exact reason is not clear. The effects of temperature on enzyme stability and activity were lower than those of known Oxds. The enzyme showed highest activity at about pH 5.5, which is close to that of Oxd of *P. chlororaphis* B23 (OxdA).[11)] The specific activity of the enzyme toward *Z*-PAOx was 24.8, which is 20 times lower than that of OxdB, OxdRE, or OxdRG,[9,10)] even when measured under anaerobic conditions by five independent experiments, but the reasons for the low activity of OxdFG remain unclear at the present time. Eighty to 95% of OxdFG activity was inhibited by the presence of 1 mM carbonyl reagents such as NH_2OH and phenylhydrazine, electron donors such as trimethylhydroquinone, *p*-phenylenediamine, and dimethoxybenzidine, and by some metal ions such as Fe^{3+}, Ag^{+}, and Cu^{+}, as seen with other Oxds.[3,6,7,9)] Enzyme activity was nearly doubled by adding thiol compounds such as DTT, mercaptoethanol, cysteine, and thioglycerol, and ferrous ions such as ferrocyanide and $FeSO_4$. As shown in Table 2, the enzyme was active toward various arylalkyl- and alkylaldoximes, converting them to the corresponding nitriles. Arylaldoximes such as benzaldoxime, pyridine-2-aldoxime, pyridine-3-aldoxime, and pyridine-4-aldoxime were inert as substrates, as was also seen with known Oxds. Table 2 also shows values for V_{max} and K_m, determined from Lineweaver-Burk plots of the kinetic data. Based on a comparison of V_{max}/K_m values, the enzyme preferentially acts on arylalkylaldoximes. Therefore, we propose tentatively that it can be categorized into a group of "phenylacetaldoxime dehydratase (EC 4.99.1.7)".

From studies on the heme environments of OxdB[10)] and OxdA,[12)] His^{306} and His^{320} residues were confirmed to be the catalytic residue located in the distal heme pocket of the Oxds, respectively, by alanine-scanning mutagenesis. As shown in Fig. 1, however, the histidine residue (boxed) is not present in OxdFG, suggesting that catalytic residues located at the distal heme pocket of OxdFG are different from those of the other Oxds. On the other hand, another histidine (boxed by a dotted line), which corresponds to His^{299} of OxdA, been proposed to be the histidine residue located in the proximal heme pocket of OxdA,[12)] is present in OxdFG. We found that the proximal histidine residue has been highly conserved among Oxds even from eukaryotes, and that it is indispensable for heme-binding. Further studies are needed to identify crucial catalytic residues of OxdFG.

Genome sequencing analysis of *F. graminearum* PH-1 revealed that the *oxd* gene exists together with genes coding for Nit (protein code: 78265.1), and most probably for its regulatory protein (protein code: 78266.1) in the genome of the strain, as we observed in *Bacillus* sp. OxB-1[3)] and *P. syringae* [AE016853].[9)] Nit of *F. graminearum* had 37.1 and 39.7% identities with those of *Bacillus* sp. and *P. syringae* respectively. A possible regulatory protein of *F. graminearum* also showed 19.6 and 18.1% identities respectively with those of the strains although they belong to different biological species: eukaryotes and prokaryotes. The characteristics of OxdFG are similar to those of OxdB in substrate specificity and subunit structure. Based on

these results, we speculate that the *oxd* gene coding for OxdFG has evolved from an ancestor gene together with genes of Nit and regulatory proteins, as we have observed in bacterial strains, in which NHase and Oxd genes are suggested to be co-evolved.[4,6,7,9)]

Mahadevan *et al.* reported some primitive studies on indoleacetaldoxime (IAOx) dehydratase (EC 4.99.1.6) of *Gibberella fujikuroi* (anamorph of *F. moniliforme*),[19)] and showed that the enzyme was activated by dehydroascorbic acid, ascorbic acid, and PLP, and was specific for IAOx. Since IAOx dehydratase has been partially purified and is poorly characterized, we cannot compare the properties of IAOx dehydratase with those of OxdFG, reported in this study. But the fact that OxdFG acts on various arylalkyl- and alkylaldoximes and is not activated by these compounds allowed us to show that OxdFG is the first example of fungal Oxd purified and characterized, and is different from the reported IAOx dehydratase. It is our interest to clarify the physiological functions of the aldoxime-nitrile pathway in the fungal strains to determine the genetic and enzymatic associations between pathogenic fungi and plants.

References

1) Asano, Y., Overview of screening for new microbial catalysts and their uses in organic synthesis: selection and optimization of biocatalysts. *J. Biotechnol.*, **94**, 65–72 (2002).
2) Kato, Y., Ooi, R., and Asano, Y., Distribution of aldoxime dehydratase in microorganisms. *Appl. Environ. Microbiol.*, **66**, 2290–2296 (2000).
3) Kato, Y., Nakamura, K., Sakiyama, H., Mayhew, S. G., and Asano, Y., A novel heme-containing lyase, phenylacetaldoxime dehydratase from *Bacillus* sp. strain OxB-1: purification, characterization, and molecular cloning of the gene. *Biochemistry*, **39**, 800–809 (2000).
4) Xie, S. X., Kato, Y., and Asano, Y., High yield synthesis of nitriles by a new enzyme, phenylacetaldoxime dehydratase, from *Bacillus* sp. strain OxB-1. *Biosci. Biotechnol. Biochem.*, **65**, 2666–2672 (2001).
5) Kato, Y., and Asano, Y., High-level expression of a novel FMN-dependent heme-containing lyase, phenylacetaldoxime dehydratase of *Bacillus* sp. strain OxB-1, in heterologous hosts. *Protein Exp. Purif.*, **28**, 131–139 (2003).
6) Xie, S.-X., Kato, Y., Komeda, H., Yoshida, S., and Asano, Y., A novel gene cluster responsible for alkylaldoxime metabolism coexisting with nitrile hydratase and amidase in *Rhodococcus globerulus* A-4. *Biochemistry*, **42**, 12056–12066 (2003).
7) Kato, Y., Yoshida, S., Xie, S.-X., and Asano, Y., Aldoxime dehydratase co-existing with nitrile hydratase and amidase in iron-type nitrile hydratase producer *Rhodococcus* sp. N-771. *J. Biosci. Bioeng.*, **97**, 250–259 (2004).
8) Kato, Y., Yoshida, S., and Asano, Y., Polymerase chain reaction for identification of aldoxime dehydratase in aldoxime- or nitrile-degrading microorganisms. *FEMS Microbiol. Lett.*, **246**, 243–249 (2005).
9) Kato, Y., and Asano, Y., Molecular and enzymatic analysis of the "aldoxime-nitrile pathway" in the glutaronitrile degrader *Pseudomonas* sp. K-9. *Appl. Microbiol. Biotechnol.*, in press.
10) Kobayashi, K., Yoshioka, S., Kato, Y., Asano, Y., and Aono, S., Regulation of aldoxime dehydratase activity by redox-dependent change in the coordination structure of the aldoxime-heme complex. *J. Biol. Chem.*, **280**, 5486–5490 (2005).
11) Oinuma, K., Hashimoto, Y., Konishi, K., Goda, M., Noguchi, T., Higashibata, H., and Kobayashi, M., Novel aldoxime dehydratase involved in carbon-nitrogen triple bond synthesis of *Pseudomonas chlororaphis* B23: sequencing, gene expression, purification, and characterization. *J. Biol. Chem.*, **278**, 29600–29608 (2003).
12) Konishi, K., Ishida, K., Oinuma, K., Ohta, T., Hashimoto, Y., Higashibata, H., Kitagawa, T., and Kobayashi, M., Identification of crucial histidines involved in carbon-nitrogen triple bond synthesis by aldoxime dehydratase. *J. Biol. Chem.*, **279**, 47619–47625 (2004).
13) Oinuma, K. I., Ohta, T., Konishi, K., Hashimoto, Y., Higashibata, H., Kitagawa, T., and Kobayashi, M., Heme environment in aldoxime dehydratase involved in carbon–nitrogen triple bond synthesis. *FEBS Lett.*, **568**, 44–48 (2004).
14) Asano, Y., Ando, S., Tani, Y., and Yamada, H., Degradation of dinitriles by *Fusarium merismoides* TG-1. *Agric. Biol. Chem.*, **44**, 2497–2498 (1980).
15) Asano, Y., Ando, S., Tani, Y., Yamada, H., and Ueno, T., Fungal degradation of triacrylonitrile. *Agric. Biol. Chem.*, **45**, 57–62 (1981).
16) Harper, D. B., Fungal degradation of aromatic nitriles. *Biochem. J.*, **167**, 685–692 (1977).
17) Malardier, L., Daboussi, M. J., Julien, J., Roussel, F., Scazzocchio, C., and Brygoo, Y., Cloning of the nitrate reductase gene (*niaD*) of *Aspergillus nidulans* and its use for transformation of *Fusarium oxysporum*. *Gene*, **78**, 147–156 (1989).
18) Sambrook, J., Fritsch, E. F., and Maniatis, T., Molecular cloning: a laboratory manual, 3rd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989).
19) Shukla, P. S., and Mahadevan, S., Indoleacetaldoxime hydro-lyase. III. Further studies on the nature and mode of action of the enzyme. *Arch. Biochem. Biophys.*, **137**, 166–174 (1970).