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High-level expression of a novel amine-synthesizing enzyme, N-substituted formamide deformylase, in *Streptomyces* with a strong protein expression system

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Abstract

N-substituted formamide deformylase (NfdA) from *Arthrobacter pascens* F164 is a novel deformylase involved in the metabolism of isonitriles. The enzyme catalyzes the deformylation of an N-substituted formamide, which is produced from the corresponding isonitrile, to yield the corresponding amine and formate. The *nfdA* gene from *A. pascens* F164 was cloned into different types of expression vectors for *Escherichia coli* and *Streptomyces* strains. Expression in *E. coli* resulted in the accumulation of an insoluble protein. However, *Streptomyces* strains transformed with a P_{nitA}-NitR system, which we very recently developed as a regulatory gene expression system for streptomycetes, allowed the heterologous overproduction of NfdA in an active form. When *Streptomyces lividans* TK24 transformed with pSH19-*nfdA* was cultured under the optimum conditions, the NfdA activity of the cell-free extract amounted to 8.5 U/mg, which was 29-fold higher than that of *A. pascens* F164. The enzyme also comprised ≈20% of the total extractable cellular protein. The recombinant enzyme was purified to homogeneity and characterized. The expression system established here will allow structural analysis and mutagenesis studies of NfdA.

Keywords: N-Substituted formamide; Deformylase; Isonitrile; *Streptomyces*; Inducible; Expression; Nitrile; Nitrilase; Promoter; Inducer; Arthrobacter; Amine; Formate

We have been interested in the enzymes involved in the metabolism of nitriles [1–5] and their isomeric forms, isonitriles. An isonitrile (more generally called an isocyanide), like a nitrile, is generally a highly toxic compound with an isocyano group (—N≡C). Although naturally occurring isonitriles are produced by various organisms, including bacteria, fungi, and marine sponges [6,7], the metabolism of isonitriles had been completely unknown at the protein and gene levels before we discovered an isonitrile-degrading enzyme, which was designated as isonitrile hydratase; it is the first known enzyme involved in isonitrile metabolism [8,9]. The enzyme catalyzes the hydration of an isonitrile to the corresponding N-substituted formamide: $R—N{\equiv}C + H_2O \rightarrow R—NH—CH(=O)$. However, there have been no reports on an enzyme that is involved in the further metabolism of an N-substituted formamide produced from the corresponding isonitrile.

Recently, we isolated a microorganism, *Arthrobacter pascens* F164, that is able to degrade *N*-benzylforma-

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mide (NBFA)[1] (which is an N-substituted formamide) and the corresponding isonitrile, benzyl isocyanide [10]. In this strain, we discovered a novel enzyme, N-substituted formamide deformylase (NfdA), that catalyzes the deformylation of an N-substituted formamide to yield the corresponding amine and formate: $R{-}NH{-}CH(=O) + H_2O \rightarrow R{-}NH_2 + HCOOH$. Among the N-substituted formamides tested, NBFA is the most suitable substrate of the enzyme, whereas the enzyme does not act on other N-substituted formamides that are substrates for other known deformylases, including kynurenine formamidase [11], formylmethionine deformylase [12], peptide deformylase [13], 10-formyltetrahydrofolate deformylase [14], formamidase [15], *N*,*N*-dimethylformamidase [16], formylaspartate deformylase [17], and formylglutamate deformylase [18]. Moreover, the deduced amino acid sequence of the gene (*nfdA*) encoding NfdA does not show any sequence identity with those of any other deformylases described above. These findings demonstrate that NfdA (which is involved in isonitrile metabolism) is a novel deformylase for N-substituted formamides, and its structure and reaction mechanism would be different from those of each of the other known deformylases. There is currently a great deal of interest in the catalytic mechanism of this enzyme. A more detailed understanding of it will be obtained by analysis of its three-dimensional structure. However, the yield of the enzyme obtained from the wild type strain was too low to perform such analyses. To increase the knowledge on the enzymology of NfdA and to shed light on the structure/function relationship of this enzyme, at first we attempted to overexpress the recombinant NfdA in various *Escherichia coli* strains as described below. Despite much effort, the NfdA was produced as inclusion bodies.

Very recently, on the other hand, we developed a novel and powerful protein expression system, designated as the P_{nitA}-NitR system, for GC-rich Gram-positive microorganisms belonging to the genus *Streptomyces* [19]. This system is based on the expression mechanism of *Rhodococcus rhodochrous* J1 nitrilase, and the expression of a target gene inserted into the expression vector, pSH19, is strongly induced by the addition of ε-caprolactam or isovaleronitrile as an inducer. Our previous studies demonstrated that the system allowed the production of target proteins, whose genes are derived from either Gram-negative or Gram-positive bacteria, in amounts corresponding to ≈40% of all soluble protein. The system was expected to be useful for the overexpression of the *nfdA* gene from *A. pascens* F164.

In this paper, we describe the heterologous overexpression of *A. pascens* NfdA in an active form by using the P_{nitA}-NitR system, and the purification of NfdA in a sufficient quantity and of a sufficient quality. Moreover, we describe the characterization of the recombinant NfdA and demonstrate that the recombinant enzyme is identical to the wild type enzyme.

Materials and methods

Materials

DEAE–Sephacel and a low-molecular-weight standard kit were obtained from Amersham Biosciences (Piscataway, NJ). Standard proteins for matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) was purchased from Sigma (St. Louis, MO, USA). *N*-Benzylformamide (NBFA) was obtained from Aldrich (Milwaukee, WI). Isovaleronitrile was from Tokyo Kasei (Tokyo, Japan). Bacto-agar was from Difco (Detroit, WI). Restriction enzymes were purchased from Takara (Tokyo, Japan), Toyobo (Osaka, Japan), and New England Biolabs (Beverly, MA), and used according to the manufacturer's directions. DNA polymerase and T4 DNA ligase were obtained from Toyobo (Osaka, Japan). All other biochemicals used were from commercial sources and of reagent grade.

Bacterial strains and plasmids

The *E. coli* strains used in this study were as follows: JM109 and DH5α were purchased from Takara; Origami B, Rosetta-gami B, Rosetta-gami B(DE3), and BL21(DE3) from Novagen; BL21(DE3) CodonPlus-RIL, JM110, and XL1-Blue from Stratagene; DH10B from Invitrogen; JM109(DE3) from Promega; and ER2566 from New England Biolabs. *Streptomyces lividans* TK24, *Streptomyces coelicolor* A3(2) M145, and *Streptomyces avermitilis* K139 were supplied by Dr. T. Fujii (National Institute for Agro-Environmental Sciences) and Prof. H. Ikeda (Kitasato University). The plasmids used and constructed in this work are shown in Table 1. The expression vector for *Streptomyces* cells, pSH19, was prepared as reported previously [19].

Recombinant DNA methods

Purification of *E. coli* plasmids and in vitro DNA manipulation for cloning into *E. coli* cells were performed as described by Sambrook et al. [20]. The

[1] *Abbreviations used:* NfdA, N-substituted formamide deformylase; NBFA, *N*-benzylformamide; P_{nitA}, nitrilase gene (*nitA*) promoter; HPLC, high-performance liquid chromatography; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.