Process Biochemistry 49 (2014) 445–450

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Cyanide hydratase from *Aspergillus niger* K10: Overproduction in *Escherichia coli*, purification, characterization and use in continuous cyanide degradation

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ARTICLE INFO

Article history:
Received 13 September 2013
Received in revised form
12 December 2013
Accepted 12 December 2013
Available online 21 December 2013

Keywords:
Cyanide hydratase
Nitrilase
Aspergillus niger
Continuous stirred membrane reactor

ABSTRACT

A cyanide hydratase from *Aspergillus niger* K10 was expressed in *Escherichia coli* and purified. Apart from HCN, it transformed some nitriles, preferentially 2-cyanopyridine and fumaronitrile. V_{max} and K_m for HCN were *ca.* 6.8 mmol min^{-1} mg^{-1} protein and 109 mM, respectively. V_{max} for fumaronitrile and 2-cyanopyridine was two to three orders of magnitude lower than for HCN (*ca.* 18.8 and 10.3 μmol min^{-1} mg^{-1}, respectively) but K_m was also lower (*ca.* 14.7 and 3.7 mM, respectively). Both cyanide hydratase and nitrilase activities were abolished in truncated enzyme variants missing 18–34 C-terminal aa residues. The enzyme exhibited the highest activity at 45 °C and pH 8–9; it was unstable at over 35 °C and at below pH 5.5. The operational stability of the whole-cell catalyst was examined in continuous stirred membrane reactors with 70-mL working volume. The catalyst exhibited a half-life of 5.6 h at 28 °C. A reactor loaded with an excess of the catalyst was used to degrade 25 mM KCN. A conversion rate of over 80% was maintained for 3 days.

1. Introduction

Nitrilases (EC 3.5.5.1) and cyanide hydratases (EC 4.2.1.66; CHTs) belong to class one of the nitrilase superfamily, which consists of enzymes acting on non-peptide C–N bonds [1]. The two enzymes differ in their substrates (nitriles *vs.* HCN, respectively) and in their major reaction products (carboxylic acids *vs.* amide, respectively).

All characterized CHTs originate from filamentous fungi, and all the genes of putative CHTs were also found in fungal genomes. CHTs are likely to play a role in the detoxification of HCN released from cyanogenic glycosides, which occur in many plant species. CHTs could have evolved from nitrilases whose genes were acquired by fungi via horizontal gene transfer from bacteria [2].

CHTs were first reported in phytopathogenic fungi (*Stemphylium loti*, *Leptosphaeria maculans*, *Gloeocercospora sorghi*, *genus Fusarium*; for a review, see [3]). Later, CHTs from saprophytic fungi *Neurospora crassa* and *Aspergillus nidulans* and from the phytopathogenic fungi *Gibberella zeae* and *G. sorghi* were expressed in *Escherichia coli* and partially characterized [4]. The genus *Aspergillus* is rich in genes coding for putative nitrilases or CHTs, but only a few of them have been characterized. Apart from the CHT from *A. nidulans*, a CHT from *Aspergillus niger* K10 (NitAn1) was expressed in *E. coli* and its activities were examined in whole cells [5]. In addition, an aromatic nitrilase with a preference for 4-cyanopyridine and benzonitrile

Abbreviations: aa, amino acid; CHT, cyanide hydratase; CSMR, continuous stirred membrane reactor; $CynD_{pum}$, cyanide dihydratase from *Bacillus pumilus*; $CynD_{stu}$, cyanide dihydratase from *Pseudomonas stutzeri*; NitAn1, cyanide hydratase/nitrilase from *Aspergillus niger* K10; NitAn1-C14, NitAn1-C18, NitAn1-C22, NitAn1-C26, NitAn1-C30 and NitAn1-C34, variants of NitAn1 missing 14, 18, 22, 26, 30 and 34 C-terminal aa residues, respectively; Nit(p), nitrilase from *Pseudomonas fluorescens* EBC 191; Nit(r), nitrilase from *Rhodococcus rhodochrous* J1; MWCO, molecular weight cut-off.

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http://dx.doi.org/10.1016/j.procbio.2013.12.008

was partially purified from a mycelium of *A. niger* K10 grown on 2-cyanopyridine [6]. This enzyme was later analyzed by mass spectrometry and found to be closely related to a hypothetical nitrilase whose gene was detected in the genome sequence of *Aspergillus kawachii* IFO 4308 [7].

In this work, NitAn1 was purified and characterized. Previously, the enzyme was expressed in *E. coli* cells [8] and found to transform not only HCN, but also selected nitriles [5]. Here the kinetics of the purified enzyme were examined with HCN and its best nitrile substrates, 2-cyanopyridine and fumaronitrile. Variants of NitAn1 truncated at the C-terminus were prepared in order to assess the effect of this region on their CHT and nitrilase activities. The operational stability of the enzyme was determined using continuous stirred membrane reactors (CSMRs), which were previously used in the transformation of nitriles *via* the monoenzymatic (nitrilase) or bienzymatic (nitrile hydratase–amidase) pathway [9,10].

2. Materials and methods

2.1. Database search and sequence alignment

Database searches were performed using the BLASTP programme (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequence alignment was performed using the COBALT: Constraint-based Multiple Alignment Tool (http://www.ncbi.nlm.nih.gov/tools/cobalt/cobalt.cgi).

2.2. Nitrilase expression and purification

NitAn1 was expressed without a His_6-tag in *E. coli* as described previously [8,11]. Briefly, the *Nde*I-*Hind*III fragment of the gene amplified from the cDNA of *A. niger* K10 was ligated into the corresponding sites of pET-30a(+) (Novagen) to give the pOK101 vector, which was then transformed into *E. coli* BL21-Gold(DE3). The cells were grown at 37 °C in Luria-Bertani medium supplemented with 50 μg mL^{-1} kanamycin until OD_{610} reached 0.4–0.6 and gene expression was then induced by adding 0.8 mM IPTG. The cells were harvested after a further 16-h cultivation at 26 °C.

Genes encoding C-terminally truncated variants missing 14, 18, 22, 26, 30 or 34 aa residues (designated NitAn1-C14, NitAn1-C18, NitAn1-C22, NitAn1-C26, NitAn1-C30 and NitAn1-C34, respectively) were prepared synthetically by Generay Biotech Co., Ltd., Shanghai, China, and expressed in the same way as the wild-type enzyme.

After disrupting the cells by sonication and removing cell debris by centrifugation (13,000 × *g*, 4 °C, 30 min), the supernatant was injected into a Hi-Prep 16/10 Q FF column (Amersham Biosciences) and the proteins eluted with a linear gradient of NaCl (0.15–1 M) in Tris/HCl buffer (50 mM, pH 8.0). Active fractions were pooled, concentrated using an Amicon Ultra-4 unit (MWCO 10 kDa) and injected into a Hi-Prep 16/60 Sephacryl S-200 column. Proteins were eluted with Tris/HCl buffer (50 mM, pH 8.0, 150 mM NaCl). Active fractions were pooled, concentrated as described above and stored at −80 °C.

2.3. Cyanide hydratase and nitrilase assays

The CHT activity was assayed in 1.5-mL Eppendorf tubes with 0.5 mL of the reaction mixtures consisting of 25 mM KCN, Tris/HCl buffer (50 mM, pH 8.0, 150 mM NaCl) and an appropriate amount of the purified enzyme or *E. coli* whole cells. The reactions proceeded with shaking (Thermomixer Eppendorf Compact, 850 rpm) at 30 °C and were stopped within their linear range (after 5 min) by the addition of methanol (final concentration 50%, v/v). The production of formamide was determined by HPLC analyses as described previously [5].

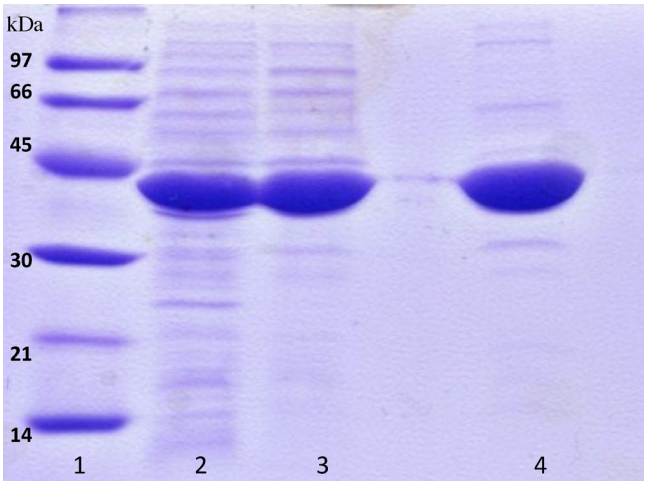

Fig. 1. Expression in *E. coli* and purification of NitAn1. Molecular weight markers (lane 1), crude extract (lane 2), pooled fractions after purification on Q-Sepharose (lane 3), pooled fractions after purification on Superdex 200 (lane 4).

The pH optimum of the purified enzyme was determined in 33 mM acetic acid/boric acid/phosphoric acid/NaOH buffer, pH 4.0–11.0, with 25 mM 2-cyanopyridine instead of HCN as the substrate, since HCN would be largely lost at a low pH. The temperature optima were determined with the same substrate in Tris/HCl buffer (50 mM, pH 8.0, 150 mM NaCl) at 25–55 °C. The pH stability was determined by measuring the residual activity for 2-cyanopyridine after pre-incubation of the enzyme in 33 mM acetic acid/boric acid/phosphoric acid/NaOH buffer, pH 4.0–11.0, at 30 °C for 2 h. The temperature stabilities were determined in the same way after pre-incubation of the enzyme in Tris/HCl buffer (50 mM, pH 8.0, 150 mM NaCl) at 25–55 °C for 1 h.

The substrate specificity of the purified enzyme was determined with 25 mM KCN, fumaronitrile, benzonitrile, 2-, 3- or 4-cyanopyridine or phenylacetonitrile in Tris/HCl buffer (50 mM, pH 8.0, 150 mM NaCl) at 30 °C. The concentrations of nitriles and their reaction products were determined by HPLC [5].

The kinetics of the purified enzyme were determined with 2–40 mM KCN or 0.5–25 mM fumaronitrile or 2-cyanopyridine under the same conditions.

2.4. Biotransformations in CSMRs

An Amicon 8050 ultrafiltration cell (Millipore, USA) with a cellulose ultrafiltration membrane (MWCO 10 kDa) was used as a CSMR with a working volume of 70 mL. The reactor was loaded with an appropriate amount of *E. coli* cells expressing NitAn1 and operated at a flow rate of 12 mL h^{-1}, 28 °C and under constant stirring (250 rpm). The stock solution of 25 mM KCN in 50 mM Tris/HCl buffer was kept under an argon atmosphere. The deactivation constant, enzyme half-life and the initial reaction rate were calculated as described previously [10].

3. Results and discussion

3.1. Substrate specificity of NitAn1

NitAn1 was purified to near homogeneity in two steps. The enzyme formed a major part of the total cellular proteins, and therefore the specific activity increased only 1.8-fold after purification, which proceeded with a yield of *ca.* 17%. SDS-PAGE analysis indicated the molecular weight of the purified protein to be *ca.* 40 kDa as expected (Fig. 1).