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# Purification and characterization of nitrilase from *Fusarium solani* IMI196840

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ABSTRACT

Nitrilase activity in *Fusarium solani* IMI196840 (approx. 1500 U $l^{-1}$ of culture broth) was induced by 2-cyanopyridine. The enzyme was purified by a factor of 20.3 at a yield of 26.9%. According to gel filtration, the holoenzyme was an approx. 550-kDa homooligomer consisting of subunits with a molecular weight of approximately 40 kDa, as determined by SDS-PAGE. Mass spectrometry analysis of the tryptic fragments suggested a high similarity of this enzyme to the hypothetical CN hydrolases from *Aspergillus oryzae*, *Gibberella zeae*, *Gibberella moniliformis* and *Nectria haematococca*. Circular dichroism and fluorescence spectra indicated that secondary structure content and overall tertiary structure, respectively, were almost identical in nitrilases from *F. solani* IMI196840 and *F. solani* O1. The melting temperatures of the enzymes were 49.3 °C and 47.8 °C, respectively. The best substrates for the purified nitrilase from *F. solani* IMI196840 were benzonitrile and 4-cyanopyridine, which were hydrolyzed at the rates of 144 and 312 U $mg^{-1}$ protein, respectively, under the optimum conditions of pH 8 and 45 °C. The enzyme was highly chemoselective, producing ≤2% amides as by-products.



## 1. Introduction

Nitrilases (EC 3.5.5.1; 3.5.5.2; 3.5.5.4–3.5.5.7) have been recognized as valuable biocatalysts for the synthesis of high added-value carboxylic acids from cheap and readily available nitriles. Interest in their use is rising as illustrated by the increasing number of articles and patents on this topic [1]. This is because the enzymes enable nitrile hydrolysis to be performed at mild pH and temperature and are often also enantioselective or regioselective. Furthermore, among nitrilases there is broad choice of enzymes with differing substrate specificities (e.g., [1–5]). A number of companies (DuPont, Lonza, Dow, Diversa, BASF, DSM) have investigated the use of enzymatic nitrile hydrolysis in their processes. For instance, (*R*)-mandelic and (*R*)-chloromandelic acid have already been produced commercially by Mitsubishi Rayon Co. [5].

Nitrilases were discovered in the 1960s, when enzymes from barley [6] and *Pseudomonas* [7] were purified. Since then, a large number of various nitrilases have been purified and characterized in bacteria, but only a few in eukaryotic organisms (plants and filamentous fungi; for a review see [3]). Nevertheless, the broad occurrence of nitrilases in filamentous fungi has been indicated by screening collection strains [8] and by gene and protein database searches [9].

We have recently been focusing our research on filamentous fungi as a source of new nitrilases with potentially different catalytic properties from those of the bacterial enzymes, and purified and characterized the nitrilases from *Aspergillus niger* K10 [10] and *Fusarium solani* O1 [11]. The latter enzyme exhibited similar specific activities to the nitrilase from *Fusarium oxysporum* f. sp. *melonis* [12]. On the other hand, the nitrilase from the *F. solani* IMI196840 strain described in one of the pioneering works on nitrilases in 1977 [13] exhibited a specific activity that was 87-times lower. This enzyme also differed from typical nitrilases in its subunit molecular mass (76 kDa [13] vs. 37–40 kDa in other fungal enzymes [10–12] and 38–46 kDa in most bacterial enzymes [3,4]).

The nitrilase in *F. solani* IMI196840 was induced by benzonitrile as the sole source of C and N but the activity yield was very low [13,14]. Previously, we identified 2-cyanopyridine as a powerful nitrilase inducer, which was efficient in all the nitrilase-producing fungal strains examined (*Aspergillus*, *Fusarium*, *Penicillium*) [15]. In this work, we used this compound to induce a high nitrilase activity in the *F. solani* IMI196840 strain. The nitrilase purified and characterized by us from this strain is substantially different from that described in the same strain previously [13] but similar to the other two nitrilases described in *Fusarium* genus [11,12], though some differences between these enzymes could be observed in terms of

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their substrate specificity, pH and temperature profiles and stability.

## 2. Materials and methods

### 2.1. Chemicals

Substrates and authentic standards of the reaction products were purchased from Alfa Aesar (Germany), Sigma–Aldrich (USA) or Merck (Germany). Chemicals for protein sequencing were purchased from Applied BioSystems Inc. (USA).

### 2.2. Microorganisms and cultivation

*Fusarium solani* IMI196840 was purchased from the CABI BIOSCIENCE Genetic Resource Collection (Egham, Surrey, UK). The strain was grown in a two-stage culture. In the first stage, the fungus was grown in shaken 500-ml Erlenmeyer flasks with 200 ml of a medium consisting of ($g l^{-1}$) sucrose 30, malt extract 5, yeast extract 5, $NaNO_3$ 2 (pH 7.3 before sterilization). After a 48-h cultivation at 28 °C the mycelium was harvested, washed with a modified Czapek-Dox medium (in $g l^{-1}$: $K_2HPO_4$ 1.0, $MgSO_4{\cdot}7H_2O$ 0.5, KCl 0.5, $FeSO_4{\cdot}7H_2O$ 0.01, $CoCl_2{\cdot}6H_2O$ 0.001, $ZnSO_4{\cdot}7H_2O$ 0.0067, yeast extract 0.1, pH 7.3 before sterilization) and resuspended in the same medium (in 200 ml/500-ml Erlenmeyer flasks). After incubating the suspension in shaken flasks for 1 h at 28 °C, 2-cyanopyridine was added to a final concentration of 3 $g l^{-1}$, and the incubation continued for a further 24 h. Afterwards the mycelium was harvested, washed with Tris/HCl buffer (50 mM, pH 8.0), frozen at −80 °C, lyophilized overnight and stored at −20 °C.

*Fusarium solani* O1 deposited in the Culture Collection of Fungi, Charles University, Prague (under accession number CCF 3635), was cultivated as described previously [11].

### 2.3. Preparation of cell-free extract

The enzyme was purified from a cell-free extract, which was obtained by grinding the lyophilized mycelium with a pestle and mortar. The homogenate was resuspended in 50 mM Tris/HCl buffer (pH 8.0) containing 0.8 M $(NH_4)_2SO_4$ and sonicated in an ultrasonic bath (2 × 5 min, 35 kHz, ELMA, Germany). After each sonication, the suspension was stirred at 4 °C for 10 min. Cell debris was removed by centrifugation (10 000 × *g*, 4 °C, 30 min).

### 2.4. Enzyme purification

The cell-free extract was diluted twice with 50 mM Tris/HCl buffer (pH 8.0) containing 0.8 M $(NH_4)_2SO_4$, centrifuged (15 000 × *g*, 4 °C, 30 min) and injected into a Hi-Prep 16/10 Phenyl FF column (low sub) (GE Healthcare, UK) pre-equilibrated with the same buffer. Proteins were eluted with a linear gradient of $(NH_4)_2SO_4$ (0.8–0 M, 40 ml) in Tris/HCl buffer (50 mM, pH 8.0) at a flow rate of 2 ml $min^{-1}$. The nitrilase was eluted at 580–200 mM $(NH_4)_2SO_4$. The active fractions were pooled and concentrated using an Amicon Ultra-4 unit (cut-off 10 kDa; Millipore, USA).

The pooled concentrated fractions from the previous step were injected into a 16/60 Hi-Prep Sephacryl S-200 column (GE Healthcare, UK) pre-equilibrated with 50 mM Tris/HCl buffer (pH 8.0) containing 150 mM NaCl and eluted with the same buffer at a flow rate of 0.6 ml $min^{-1}$. The active fractions were pooled and concentrated as described above.

The pooled concentrated fractions from the previous step were diluted fivefold with 50 mM Tris/HCl buffer, pH 8.0, and injected into a Hi-Prep 16/10 Q FF column (GE Healthcare, UK). Proteins were eluted with a linear gradient of NaCl (0–1 M, 40 ml) in 50 mM Tris/HCl buffer (pH 8.0) at a flow rate of 2 ml $min^{-1}$. The nitrilase was eluted at 280–450 mM NaCl. The active fractions were pooled, concentrated as described above and sucrose was added to a concentration of 2%. The purified enzyme was stored at −80 °C.

### 2.5. Analytical size exclusion chromatography

A TSK G3000SW column (Tosoh Bioscience, Germany) was used to determine the molecular mass of the native protein. The column was equilibrated with 50 mM Na/K phosphate buffer, pH 7.0, 150 mM NaCl and the flow rate was 1.5 ml $min^{-1}$. A gel filtration HMW calibration kit (GE Healthcare, UK) for the range 158–669 kDa was used for calibration.

### 2.6. Electrophoresis

SDS polyacrylamide gel electrophoresis was performed according to Laemmli [16] in 12% polyacrylamide slab gels with protein molecular weight standards in the range of 14.4–97 kDa (GE Healthcare, UK).

### 2.7. Protein assay

The amount of protein was determined according to Bradford [17] using bovine serum albumin as the standard.

### 2.8. Mass spectrometry analysis

The protein band of the purified enzyme was manually excised from the SDS polyacrylamide gel. After destaining and washing (acetonitrile and water), the digestion with trypsin (50 μg $ml^{-1}$) was performed in 50 mM 4-ethylmorpholine acetate buffer (pH 8.1) overnight at 37 °C. The tryptic peptides were extracted and analyzed after desalting with a UltraFLEX III MALDI-TOF/TOF mass spectrometer (Bruker-Daltonics) and identified based on peptides LIFT spectra using the MS/MS ion search of Mascot program (Matrix Science) or *de novo* sequencing. Homologous proteins were searched for in the NCBI database using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/).

### 2.9. CD and fluorescence spectroscopy

Circular dichroism spectra were recorded at room temperature (22 °C) using a Jasco J-810 spectropolarimeter (Jasco, Japan). Data were collected from 185 to 260 nm, at 100 nm $min^{-1}$, 1 s response time and 2 nm bandwidth using a 0.1 cm quartz cuvette containing the enzyme in 50 mM potassium phosphate buffer (pH 7.5). Each spectrum was the average of ten individual scans and was corrected for absorbance caused by the buffer. Collected CD data were expressed in terms of the mean residue ellipticity ($\Theta_{MRE}$) using the equation:

$$\Theta_{MRE} = \frac{\Theta_{obs} M_w 100}{ncl}$$

where $\Theta_{obs}$ is the observed ellipticity in degrees, $M_w$ is the protein molecular weight, $n$ is number of residues, $l$ is the cell path length, $c$ is the protein concentration (0.22 and 0.25 mg $ml^{-1}$ for nitrilases from *F. solani* O1 and *F. solani* IMI196840, respectively) and the factor 100 originates from the conversion of the molecular weight to mg $dmol^{-1}$. Secondary structure content was evaluated from the spectra using CD Spectra Deconvolution program [18] and Self Consistent method [19] implemented in the program DICROPROT [20,21]. Thermal unfolding of the enzymes was followed by monitoring the ellipticity at 221 nm over the temperature range of 20–80 °C, with a resolution of 0.1 °C, at a heating rate of 1 °C $min^{-1}$. Recorded thermal denaturation curves were roughly normalized to represent signal changes between approximately 1 and 0 and fitted to sigmoidal curves using software Origin 6.1 (OriginLab, Massachusetts, USA). The melting temperatures ($T_m$) were evaluated as the midpoint of the normalized thermal transition.

Fluorescence spectra were acquired by using spectrofluorimeter FluoroMax-4P (HORIBA Jobin Yvon, USA) at room temperature (22 °C) from 285 to 450 nm. Fluorescence emission spectra were measured with an excitation bandwidth of 1 nm, an emission bandwidth of 1 nm; and scan speed of 50 nm $min^{-1}$ using a 0.5 cm quartz cuvette. The excitation wavelength was 280 nm. The sample concentrations were same as used for CD measurements. Each spectrum was corrected for fluorescence caused by the buffer.

### 2.10. Nitrilase assays

#### 2.10.1. Spectrometric method

A rapid, semiquantitative detection of the nitrilase activity in fractions obtained by purification steps was performed by monitoring the absorption of benzoic acid at 238 nm ($\varepsilon$ = 3300 $M^{-1}$ $cm^{-1}$) with a Saphire$^2$ plate reader (Tecan, USA). The reaction proceeded on UV-transparent 96-well plates (Nunc, USA) containing 15 μl of 10 mM benzonitrile in methanol, 250 μl Tris/HCl (50 mM, pH 8) and 5 μl of the enzyme solution at 45 °C. The reaction was quenched after 5 min by the addition of 30 μl of 1 M HCl. A reaction mixture with the same amount of HCl added at zero reaction time was used as the blank.

#### 2.10.2. HPLC method

The nitrilase activity was assayed with 25 mM benzonitrile (from 500 mM stock solution in methanol) in 50 mM Tris/HCl (pH 8.0) at 45 °C. The reaction was started by the addition of substrate after 5-min preincubation at 45 °C and quenched after 10 min with 1 M HCl (0.01 ml/0.1 ml of the reaction mixture). The activity of the enzyme in the presence of inhibitors and cosolvents was also assayed under these conditions in reaction mixtures containing various metal ions, cosolvents or other additives.

The substrate specificity was assayed with a 25 mM concentration of various nitriles under the same conditions except for bromoxynil and ioxynil, which were examined at 0.5 mM concentrations (due to their low solubility) and prolonged reaction times (30 min), after which methanol (0.1 ml/0.1 ml of the reaction mixture) was added instead of HCl to stop the reaction, while avoiding substrate precipitation. Optimum pH was determined using 50 mM Britton–Robinson (acetic acid/boric acid/phosphoric acid/NaOH) buffers (pH 4–12) at 45 °C. Optimum temperature was determined for reactions performed at pH 8.0 (50 mM Tris/HCl buffer) and various temperatures (25–60 °C). The concentrations of benzonitrile, its analogues and the corresponding reaction products (acids, amides) were analyzed using a Chromolith Flash RP-18 (Merck; 25 mm × 4.6 mm) in a mobile phase consisting of acetonitrile (10–25%, v/v) in water and 0.1% (v/v) $H_3PO_4$ at a flow rate of 2 ml $min^{-1}$ and 35 °C. Heterocyclic nitriles and their products were analyzed as described previously [22]. Results of enzymatic assays were calculated from four independent measurements.