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ORIGINAL PAPER

Hyperinduction of nitrilases in filamentous fungi

Ondřej Kaplan · Vojtěch Vejvoda · Andrea Charvátová-Pišvejcová · Ludmila Martínková

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Abstract 2-Cyanopyridine proved to act as a powerful nitrilase inducer in *Aspergillus niger* K10, *Fusarium solani* O1, *Fusarium oxysporum* CCF 1414, *Fusarium oxysporum* CCF 483 and *Penicillium multicolor* CCF 2244. Valeronitrile also enhanced the nitrilase activity in most of the strains. The highest nitrilase activities were produced by fungi cultivated in a Czapek-Dox medium with both 2-cyanopyridine and valeronitrile. The specific nitrilase activities of these cultures were two to three orders of magnitude higher than those of cultures grown on other nitriles such as 3-cyanopyridine or 4-cyanopyridine.

Keywords Nitrilase · Hyperinduction · *Aspergillus* · *Fusarium* · *Penicillium*

Introduction

Bacterial nitrilases, acknowledged as useful biocatalysts for the mild hydrolysis of nitriles, have been thoroughly described [1]. On the other hand, there are few publications on nitrilases from filamentous fungi [1]. Two nitrilases have been purified from fungi belonging to the genus *Fusarium* [2, 3]. The nitrilases of filamentous fungi seem to be worth an intensive study, as they represent useful tools for the preparation of valuable (hetero) aromatic carboxylic acids from nitriles. A study monitoring the occurrence of aldoxime dehydratases in microorganisms revealed that these enzymes were accompanied by nitrilases in all examined strains belonging to 17 genera of filamentous fungi [4]. A screening of about one hundred strains of filamentous fungi performed by us also suggested a broad distribution of nitrilases in these organisms. However, these strains belonging to *Aspergillus*, *Fusarium* and *Penicillium* produced low levels of nitrilases [5], which could not compete with the production of these enzymes in bacteria. This encouraged us to perform a screening for more powerful inducers of the nitrilase activity in these filamentous fungi. Herein, we report on the hyperinduction of this enzyme in various filamentous fungi by 2-cyanopyridine and valeronitrile.

O. Kaplan · V. Vejvoda · A. Charvátová-Pišvejcová · L. Martínková (✉)
Laboratory of Biotransformation, Institute of Microbiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, 142 20 Prague 4, Czech Republic
e-mail: martinko@biomed.cas.cz

Materials and methods

Chemicals

The following nitriles were examined as inducers of the nitrilase activity: acetonitrile (>99.9%) from Merck, Darmstadt, Germany; butyronitrile (>99%), benzonitrile (99%), 2-cyanopyridine (99%), 3-cyanopyridine (98%), picolinamide (98%), all from Sigma-Aldrich, Steinheim, Germany; 4-cyanopyridine (>98%), isobutyronitrile (>98%), propionitrile (>99%) and valeronitrile (>98%), all from Merck-Schuchardt, Hohenbrunn, Germany.

Microorganisms and cultivation

Aspergillus niger K10 and *Fusarium solani* O1 were as described previously [5, 6]. The strains are deposited in

the culture collection of fungi (CCF), Charles University Prague, Czech Republic. *Fusarium oxysporum* CCF 1414, *Fusarium oxysporum* CCF 483 and *Penicillium multicolor* CCF 2244 originated from the same culture collection. All microorganisms were maintained on a modified Czapek-Dox medium (in g/l: sucrose 30, 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) supplemented with 3-cyanopyridine (20 mM) as the sole nitrogen source and agar (15 g/l). Submerged cultivation proceeded in modified Czapek-Dox medium supplemented with different sources of nitrogen: (i) $NaNO_3$ (2 g/l) and acetonitrile, propionitrile, butyronitrile, isobutyronitrile, valeronitrile, 2-cyanopyridine, 3-cyanopyridine, 4-cyanopyridine or picolinamide (20 mM each), (ii) $NaNO_3$ (2 g/l) and 3-cyanopyridine (15 mM), (iii) $NaNO_3$ (2 g/l) and 2-cyanopyridine (15 mM), (iv) 2-cyanopyridine (15–20 mM) and (v) 2-cyanopyridine (15–20 mM) and valeronitrile (2.5–7.5 mM). Variations in sucrose concentration (10, 15 and 20 g/l) were examined using the medium with 2-cyanopyridine (20 mM) and valeronitrile (2.5 mM). Media (100 ml per 500 ml Erlenmeyer flask) were inoculated with fungal spores or mycelial suspensions and incubated at 28°C in an orbital shaker for 40–72 h.

Preparation of cell extracts

The mycelium was filtered off, washed with Tris/HCl (50 mM, pH 8) and ground to powder in a mortar. The mycelium powder was suspended in the above buffer (approx. 3 ml of buffer per 1 g of wet mycelium) and stirred for 20 min in an ice bath. The pellet was removed by centrifugation at 13,000*g* for 30 min at 4°C. The supernatant was used for the nitrilase assay (see below).

Enzyme assay

The reaction mixture (total volume of 0.1 ml) consisting of Tris/HCl (50 mM, pH 8), 25 mM benzonitrile and the cell extract (approx. 20–100 μg of protein) was shaken at 850 rpm and 30°C (for *F. oxysporum* and *P. multicolor*) or 45°C (for *A. niger* and *F. solani*) in Thermomixer Compact Eppendorf for 10 min. The reaction was stopped by the addition of 1M HCl (0.01 ml) and the precipitated protein was removed by centrifugation. The supernatant was analyzed for benzonitrile, benzoic acid and benzamide by HPLC as described previously [6]. One unit of the nitrilase was expressed as the amount of enzyme that forms 1 μmol of total product (benzoic acid plus benzamide) from benzonitrile per 1 min under the above conditions. The relative activities of the cell extracts for 2-, 3- and 4-cyanopyridine (25 mM each) were assayed under the same conditions.

Analysis of culture media

The filtrates of culture media were acidified with 1 M HCl (0.1 ml per 1 ml of sample) and centrifuged to remove extracellular proteins. The supernatant was analyzed for 2-cyanopyridine, 3-cyanopyridine and the corresponding biotransformation products by reversed-phase HPLC as described previously [6]. Valeric acid was determined by ion exchange HPLC using a Polymer IEX-H^+ column (250 × 8 mm, 8 μm; Watrex Prague, Czech Republic) and a mobile phase consisting of 9 mM sulfuric acid at a flow rate of 0.5 ml/min.

Protein assay

Protein was determined according to Bradford [7] using bovine serum albumin as the standard.

Results

Comparison of the ability of different nitriles to induce a nitrilase in *Aspergillus niger*

Previously, we reported on a nitrilase activity of *Aspergillus niger* K10 grown on 3-cyanopyridine. According to Kobayashi and Shimizu [8], the enzyme was classified as an aromatic nitrilase. Whole cells and cell extracts of this fungus were used for mild nitrile hydrolysis [6]. Nevertheless, their nitrilase activity was significantly lower than that of bacteria [9, 10]. Therefore, other nitriles were tested as potential inducers of the nitrilase activity in *A. niger*. The activity assay was performed with the cell extract to ensure an accurate enzyme dosage in each reaction mixture.

No nitrile supported fungal growth when used as the sole source of nitrogen. Therefore, both sodium nitrate and different nitriles (20 mM each) were added to the modified Czapek-Dox medium in all experiments summarized in Table 1.

The low nitrilase activity induced by 3-cyanopyridine was consistent with the previous observation [6]. All aliphatic nitriles except acetonitrile were better inducers than 3-cyanopyridine. In particular, valeronitrile induced a remarkable activity increase (by about 34-fold). Further, the induction effect of 3-cyanopyridine was compared to that of its isomers. 4-Cyanopyridine was a very poor nitrilase inducer. On the other hand, 2-cyanopyridine was a nitrilase inducer of choice,

Table 1 Protein and nitrilase production by *Aspergillus niger* grown with different nitriles

Note: *A. niger* was cultivated in a modified Czapek-Dox medium with $NaNO_3$ (2 g/l) and various nitriles (20 mM each) for 65 h. See Materials and methods for details

[a] Benzonitrile

Nitrile (20 mM each)	Protein (mg/l culture)	Specific activity[a] (U/mg protein)	Total activity (U/l culture)
Acetonitrile	113	0	0
Propionitrile	134	0.05	6.7
Butyronitrile	125	0.02	2.5
Isobutyronitrile	90	0.01	0.9
Valeronitrile	83	0.24	19.9
2-Cyanopyridine	25	2.83	71.8
3-Cyanopyridine	129	0.007	0.9
4-Cyanopyridine	150	0.002	0.3

increasing the specific activity by about 400-fold. The total activity induced by 2-cyanopyridine was also the best, though the fungal growth was reduced by this compound.

Most 2-cyanopyridine (14.4 mM) remained unconsumed after 65-h cultivation. The rest of the compound was converted into picolinic acid (1.5 mM) and picolinamide (3.3 mM).

3-Cyanopyridine was converted to a similar extent (by about 30%), the only product being nicotinic acid (5.5 mM). The observation that different types of products (amide plus acid vs only acid) were produced from the superior and inferior inducers, respectively, suggested that picolinamide might act as the actual inducer. However, picolinamide (20 mM) did not induce any nitrilase activity when added to the culture medium instead of 2-cyanopyridine.

An even higher nitrilase activity was produced by *A. niger* grown in the presence of both 2-cyanopyridine (15 mM) and valeronitrile (7.5 mM) (see Table 2). The specific activity reached its maximum after 48 h and decreased slowly at later cultivation stages. However, the total activity increased up to 65 h due to the constant increase of extractable protein (see Fig. 1). In this medium, 2-cyanopyridine was also transformed into picolinic acid (0.1 mM) and picolinamide (0.4 mM). No biotransformation products of valeronitrile were found.

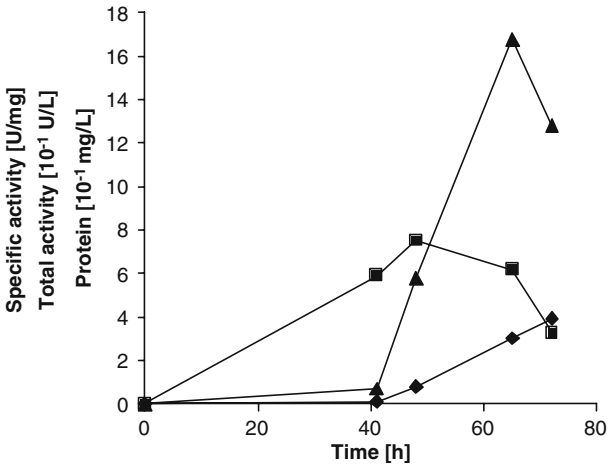

Fig. 1 Specific (*filled square*) and total activity (*filled triangle*) of nitrilase and extractable protein (*filled diamond*) in *Aspergillus niger* K10 grown in a Czapek-Dox medium containing 15 mM 2-cyanopyridine and 7.5 mM valeronitrile. The enzyme assay was performed with benzonitrile. See Materials and methods for details

Nitrilase induction in strains belonging to the genera *Fusarium* and *Penicillium*

Four other nitrile-hydrolyzing strains of filamentous fungi were grown in the same way. The nitrilase activities of *Aspergillus niger* and *Fusarium solani* were determined at 45°C, as this temperature was previously found to be close to the enzyme optimum [5]. However, the nitrilases from both strains of *F. oxysporum* and *P. multicolor* were inactivated at this temperature. Therefore, the assays were performed at 30°C. The relative activities of all strains belonging to the genus *Fusarium* were similar as those of *A. niger* K10 (see Table 2): both specific and total activities were the lowest in the cells grown on 3-cyanopyridine and sodium nitrate and the highest in the cells grown on 2-cyanopyridine and valeronitrile. *P. multicolor* exhibited the highest activity (both specific and total) when cultivated on 2-cyanopyridine and sodium nitrate. *F. oxysporum* CCF 1414 gave the best activities of all the tested fungi: 11.4 U/mg protein and 120 U/l of the culture.

Most of the fungal nitrilases produced benzamide as a by-product from benzonitrile. Benzamide formation was especially pronounced for the enzyme from *A. niger* K10. This might suggest that this fungus produced a nitrile hydratase and an amidase. However, this hypothesis was negated by observations that the ratio of benzoic acid and benzamide were very similar under different reaction conditions (pH, temperature) and during partial purification of the enzyme [11].

In *F. oxysporum* strains, the nitrilases induced by 2-cyanopyridine produced some benzamide. However, this compound could be hardly detected, when using extracts from the same strains grown on 3-cyanopyridine.

Table 2 Protein and nitrilase production by filamentous fungi grown in different media

Microorganism	Medium	Protein (mg/l culture)	Specific activity[a] (U/mg protein)	Total activity[a] (U/l culture)	Benzamide (% of total product)
Aspergillus niger K10	A	26.0	0.17	4.4	7.4
	B	5.0	6.8	34.2	7.4
	C	66.7	0.003	0.2	9.7
Fusarium oxysporum CCF 1414	A	49.0	2.1	103.9	4.3
	B	10.5	11.4	119.7	4.5
	C	65.0	0.02	1.3	0
Fusarium oxysporum CCF 483	A	24.4	0.27	6.6	4.0
	B	2.8	6.2	17.6	4.0
	C	45.0	0.04	1.8	0
Fusarium solani O1	A	40.0	0.08	3.2	0
	B	10.5	3.2	34.0	0.3
	C	45.0	0.02	0.9	0
Penicillium multicolor CCF 2244	A	19.4	0.48	9.3	2.1
	B	17.9	0.14	2.5	1.8
	C	60.0	0.01	0.6	5.6

Note: Each fungus was cultivated in a modified Czapek-Dox medium with (A) 2-Cyanopyridine (15 mM) and $NaNO_3$ (2 g/l), (B) 2-Cyanopyridine (15 mM) and valeronitrile (7.5 mM) and (C) 3-Cyanopyridine (15 mM) and $NaNO_3$ (2 g/l) for 40-45 h. See Materials and methods for details

[a] Benzonitrile

This may indicate that different isoenzymes were induced under different conditions. However, an exact determination of the minor product was difficult due to the low activity of these extracts.

The products of 2-cyanopyridine biotransformation in the culture medium were the same as with *A. niger*. The strain with the highest nitrilase activity (*F. oxysporum* CCF 1414) produced also the highest concentrations of picolinic acid (1.5 mM) and particularly picolinamide (6 mM). This culture, as well as *F. oxysporum* CCF 483, also produced valeric acid (0.9 and 1.4 mM, respectively) from valeronitrile.

Effect of culture medium on the nitrilase production by *F. solani* O1

The nitrilase activity of *F. solani* was more thermostable than that of both strains of *F. oxysporum*. In addition, this enzyme did not produce by-products (amides) as that from *A. niger* [5, 6]. However, the specific activity of the extract was lower than that of *F. oxysporum* and *A. niger*. Therefore, attempts at increasing the nitrilase activity in *F. solani* were made by varying the concentration of inducers (2-cyanopyridine, valeronitrile) and sucrose (see Table 3). The initial medium contained 15 mM of 2-cyanopyridine as the sole source of nitrogen. Contrary to *A. niger* K10, *F. solani* was able to grow on this medium. An overall improvement of the specific activity by more than 100% (up to 5.3 U/mg) was achieved within this optimization procedure, the optimum medium containing 15 g/l of sucrose, 20 mM of 2-cyanopyridine and 2.5 mM of valeronitrile. Nevertheless, the total activity was not substantially increased in this medium. The highest total activity (91 U/l) has been obtained when using a medium with 20 mM 2-cyanopyridine and 5 mM valeronitrile.

Substrate specificity of fungal nitrilases

Cell extracts from strains belonging to *Aspergillus* and *Fusarium* were examined for their activity towards cyanopyridines, which were previously reported as substrates of fungal nitrilases [2, 3, 5, 6]. The strains of *F. oxysporum* hydrolyzed 3-cyanopyridine and 4-cyanopyridine with relative activities of approx. 40 and 60%, respectively, as compared with benzonitrile. 2-Cyanopyridine was an inferior substrate of these strains, being transformed at relative activities <5%. Neither was this compound accepted by the enzyme from *F. solani*, as also observed for whole cells [5]. The highest relative activity for this compound (10% of that for benzonitrile) was found for the enzyme from *A. niger*. High relative activities towards 4-cyanopyridine were typical for the enzymes from *F. solani* and *A. niger* (approx. 250 and 350% of those for benzonitrile). The relative activities of the extracts obtained from the medium with 2-cyanopyridine and valeronitrile and that with 2-cyanopyridine and sodium nitrate exhibited no significant differences. On the other hand, the relative activities of the enzymes induced by 3-cyanopyridine could not be calculated exactly due to very low conversion rates of some substrates, especially 2-cyanopyridine.

Table 3 Nitrilase production by *Fusarium solani* O1 grown in media with different concentrations of nitriles and sucrose

Sucrose (g/l)	2-Cyanopyridine (mM)	Valeronitrile (mM)	Specific activity[a] (%)	Total activity[b] (%)
30	15	0	100	100
30	20	0	119	65
30	20	7.5	118	84
30	20	5	114	173
30	20	2.5	154	123
20	20	2.5	170	100
15	20	2.5	213	113
10	20	2.5	211	113

Note: The fungus was cultivated in a modified Czapek-Dox medium for 42 h. See Materials and methods for details

[a] The specific activity of 2.5 U/mg protein was taken as 100%

[b] The total activity of 52.8 U/l culture was taken as 100%

Discussion

Aromatic nitrilases were efficiently induced in several species of filamentous fungi. These enzymes could be roughly divided into two distinct types. The enzyme from *F. oxysporum* was inactivated at 45°C, while the enzymes from *F. solani* and *A. niger* showed optimum activities at these temperatures. In addition, remarkable differences were found between both types in their relative activities towards benzonitrile and 4-cyanopyridine. Thus the enzyme from *F. solani* O1 showed similar properties as the nitrilase purified from *F. solani* by Harper [3]. Nevertheless, there is a striking difference between the specific activities of these two enzymes. While the purified enzyme showed a specific activity of 1.66 U/mg, the specific activity of the cell extract obtained in this work was three times higher. With regard to the remarkable activities of cell extracts, we can expect high specific activities for the purified nitrilases. This is in accordance with the data reported for the purified nitrilase from *F. oxysporum f.* sp. *melonis* (143 U/mg protein) [2], as well as with our findings that the specific activity of *A. niger* K10 was increased to about 92 U/mg by partial purification [11]. It is possible that the activity of the enzyme from *F. oxysporum* CCF 1414 could be even higher than that of the nitrilase from the same species [2]. The very high activity of the cell extract from this strain (11.4 U/mg protein) supports this expectation.

Hyperinduction of an aromatic nitrilase has been demonstrated in *Rhodococcus rhodochrous* J1 [12]. The superior inducer of the nitrilase in this bacterium was isovaleronitrile. The same inducer was highly efficient for the induction of an aliphatic nitrilase in *Rhodococcus rhodochrous* K22 [13]. In both bacteria, the nitrilase activities of the cultures were remarkably affected by the type of the nitrile added to the culture medium. Depending on the inducer, the range of nitrilase activity was 0.1–2.1 and 0.02–12.3 U/mg of dry cell weight for the former and latter strain, respectively. In *Fusarium oxysporum f.* sp. *melonis* [2], the differences between the nitrilase activities of cells induced by different nitriles [acetonitrile, propionitrile, (iso) butyronitrile, benzonitrile] were not so striking (0.1–0.53 U/mg of dry cell weight). No comparison of different inducers has been provided for *Fusarium solani*, which was grown on benzonitrile as the sole source of carbon and nitrogen [3].

Valeronitrile, which proved to act as a good inducer of nitrilases in filamentous fungi examined in this work, has not been tested in the above works dealing with fungal nitrilases. In *R. rhodochrous* J1, it did not induce any nitrilase activity (similarly as other aliphatic nitriles), while branched nitriles (isovaleronitrile and also isobutyronitrile and isocapronitrile) were very good inducers [12]. 2-Cyanopyridine, which was selected in the present work as the best inducer of nitrilase activity, has not been tested for the above fungal nitrilases. Though a nitrilase activity has been induced by 2-cyanopyridine in *Nocardia rhodochrous* LL100–21, no significant difference was observed between the inducing effect of this compound and that of 3-cyanopyridine [9]. In the present work, the difference in the nitrilase activity between 3-cyanopyridine- and 2-cyanopyridine-induced cultures of *A. niger* was striking (0.007 and 2.83 U/mg of protein).

In conclusion, a new powerful inducer of aromatic nitrilases has been found. The hyperinduction of nitrilases by this nitrile makes nitrile-converting fungi competitive with bacterial nitrile-hydrolyzing biocatalysts. The induction of nitrilases in different species of filamentous fungi seems to follow a similar pattern and thus it appears to be of wide potential use.

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