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Biocatalytic application of nitrilases from *Fusarium solani* O1 and *Aspergillus niger* K10

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ABSTRACT

The nitrilases from *Fusarium solani* O1 and *Aspergillus niger* K10 showed a broad substrate specificity for carbocyclic and nonaromatic heterocyclic amino nitriles, the preferred substrates being five-membered γ-amino nitrile (±)-**1a**, six-membered γ-amino nitriles (±)-**3a**, (±)-**5a** and (±)-**6a**, pyrrolidine-3-carbonitriles (±)-**9a** and (±)-**10a** as well as piperidine-4-carbonitriles **14a** and **15a**. Both enzymes showed a strong diastereopreference for *cis*- vs. *trans*-γ-amino nitriles. The electronic and steric effects of *N*-protecting groups affected the reactivity of the nitriles. Amides as by-products of the nitrilase-catalyzed reaction were produced from heterocyclic amino nitriles (±)-**9a**, (±)-**10a**, **14a** and **15a** by the *A. niger* enzyme but only from nitrile (±)-**9a** by the *F. solani* enzyme.

1. Introduction

Since their discovery in 1964 [1,2] nitrilases have been reported in many organisms: bacteria, fungi and plants (for a review see Ref. [3]). The biocatalytic impact of nitrilases resides in their ability to hydrolyze a broad spectrum of synthetic nitriles. These enzymes can be divided into three types according to their substrate specificity, that is aromatic, aliphatic and arylaliphatic nitrilases [4].

The importance of filamentous fungi as a source of new nitrilases has been fully acknowledged just recently, when new information on this group of enzymes was presented in several studies [5–10]. In general, the purified and characterized nitrilases from *Fusarium solani* [9,11], *Fusarium oxysporum* f. sp. *melonis* [12] and *Aspergillus niger* [5] belong to aromatic nitrilases and they largely show high specific activites for the corresponding substrates (benzonitrile, its analogues and cyanopyridines). These enzymes are in most cases chemoselective, that is they produce little of the nitrilase by-product amide [9–12], and regioselective, e. g., for benzenedicarbonitriles [5] and pyridinedicarbonitriles [8].

The aim of the present work was to get new information on the biocatalytic potential of the recently purified nitrilases from *F. solani* O1 [9] and *A. niger* K10 [5], namely on the ability of these enzymes to transform branched and substituted nitriles. Therefore, we carried out screening of their activities as well as stereo- and chemoselectivities towards a series of carbocyclic and nonaromatic heterocyclic amino nitriles. Recently, these nitriles were synthesized and their hydrolysis was catalyzed by the commercial nitrilases from Biocatalytics, Inc. [13,14]. This enabled to compare the catalytic properties of the new fungal nitrilases with a set of commercial nitrilases with widely varying specificities and selectivities.

2. Experimental

2.1. Microorganisms

A. niger K10 and *F. solani* O1 are deposited in the Culture Collection of Fungi, Charles University Prague, Czech Republic (under accession numbers CCF 3411 and CCF 3635, respectively). The mycelia were grown in submerged cultures with 2-cyanopyridine (nitrilase inducer) as described previously [5,9]. After being harvested, the mycelium of *F. solani* was frozen at −70 °C and lyophilized overnight.

2.2. Enzyme purification

The nitrilases were purified from mycelia of *F. solani* and *A. niger* as described previously [5,9].

2.3. Substrates

The substrates were prepared and characterized as described previously [13,14].

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2.4. Typical biotransformation experiment

For screening experiments the purified enzymes from *F. solani* (approx. 4 μg of protein) and *A. niger* (approx. 6 μg of protein), i.e. 0.6 U as determined with benzonitrile, were diluted to 99.5 μL with phosphate buffer (50 mM, pH 8.0, 1 mM EDTA). The solution of the substrate in DMSO (40 mM, 0.5 μL) was added to give a final concentration of 0.2 mM. The reactions proceeded at 30 °C in a Thermomixer at 1100 rpm. After 18 h, 100 μL of acetone was added. The reaction vessels were centrifuged at room temperature and 13,000 rpm for 5 min to remove the biomass. The supernatants were analyzed by RP-18 HPLC as described below. For *ee*-determination the products were extracted using ethyl acetate. The solvent was removed and the residue dissolved in MeOH prior to chiral HPLC analysis.

For screening experiments with lyophilized mycelium of *F. solani*, 2.0 mg of the latter (0.9 U, determined with benzonitrile) was suspended in 497.5 μL of phosphate buffer (50 mM, pH 8.0, 1 mM EDTA). The solution of the substrate in DMSO (40 mM, 2.5 μL) was added to give a final concentration of 0.2 mM. The reactions proceeded at 30 °C in a Thermomixer at 1100 rpm. After 18 h, 500 μL of acetone was added and samples were prepared and analyzed as those from experiments with purified enzymes.

2.5. Analytical HPLC

Analytical HPLC was conducted with an Agilent Series 1100 HPLC using a G1315A diode array detector. For determination of the conversions a LiChrospher 100 RP18e column (5 μm) was used at ambient temperature. The compounds were separated with a gradient of 0.1% H_3PO_4 (phase A) and acetonitrile (phase B) that started with 20% phase B (5 min), increased linearly to 70% phase B over 15 min and was held at 70% phase B for 2 min. The flow rate was 0.8 ml min^{-1}. Enantiomeric excesses were determined on a Daicel Chiralpak AD-H (5 μm) column using *n*-heptane/*i*-propanol 1:1 at 0.5 ml min^{-1} and 20 °C (**1a**), *n*-heptane/EtOH 1:1 at 0.5 ml min^{-1} and 20 °C (**9a**, **10a**) and *n*-heptane/EtOH 4:1 at 0.8 ml min^{-1} and 20 °C (**5a**).

3. Results and discussion

3.1. Substrate specificity

The substrate specificity of the nitrilases from *F. solani* and *A. niger* was examined with a series of *trans*- and *cis*-configured carbocyclic nitriles, as well as nonaromatic heterocyclic nitriles. The former enzyme was used as both the purified protein and the lyophilized mycelium, but the latter one only as a partially purified enzyme due to instability of the nitrilase activity in the lyophilized cells of *A. niger*. Both enzymes were previously characterized as aromatic nitrilases but they also showed moderate activities on straight chain aliphatic nitriles (the best substrate of this type being valeronitrile transformed at 20–26% of the reference rate for benzonitrile). Only few publications suggested that substrates other than (hetero)aromatic nitriles could be hydrolyzed by some aromatic nitrilases, e.g., aliphatic nitriles and dinitriles by enzymes of *Rhodococcus rhodochrous* NCIMB 11216 [16–18] and *Fusarium oxysporum* f. sp. *melonis* [12]. However, efficient hydrolysis of branched nitriles by aromatic nitrilases has been rarely demonstrated, e.g., for 2-methylbutyronitrile [16,18], 2-chlorobutyronitrile, 2-bromobutyronitrile and 2-methylhexanenitrile [18]. The enzyme from *F. solani* O1 also showed low activities for branched and substituted aliphatic nitriles such as lactonitrile, isobutyronitrile, 2-chloropropionitrile and 2-phenylpropionitrile [9]. The *A. niger* nitrilase hydrolyzed such nitriles (albeit at very low rates) as well, e.g., 2-phenylpropionitrile [5], 2-(2′-methoxyphenyl)-propionitrile, 2-(3′-methoxyphenyl)-propionitrile [15] and 2-chloropropionitrile.

The data in Table 1 reflect the ability of the purified nitrilase from *F. solani* to hydrolyze branched aliphatic nitriles such as carbocyclic nitriles (±)-**1a**, (±)-**3a**, (±)-**5a** and (±)-**6a** and nonaromatic heterocyclic nitriles (±)-**9a**, (±)-**10a**, **14a** and **15a** effectively. In addition, carbocyclic nitriles (±)-**4a** and (±)-**7a** as well as heterocyclic nitriles (±)-**12a** and (±)-**13a** were also transformed by this enzyme, albeit at low rates. The same substrates were hydrolyzed by the purified nitrilase from *A. niger* except for two of the poor substrates, (±)-**4a** and (±)-**12a** (see Table 1). Thus, the fungal nitrilases exhibited very close substrate specificities with respect to the nitriles shown in Fig. 1. The previous screening for (hetero)aromatic substrates of these nitrilases also gave similar results for both enzymes except for a few benzonitrile analogues [5,9].

From the set of nitrilases available from Biocatalytics only a few enzymes, especially NIT-104, NIT-106 and NIT-107, were able to transform these nitriles efficiently [13,14]. Using the fungal enzymes (6 U ml^{-1}), comparable conversions were often achieved after 18 h reaction compared to commercial nitrilases applied at similar or higher enzyme loads (in U ml^{-1} of the reaction mixture NIT-104; 7.4, NIT-106; 170, NIT-107; 5.6). The substrate specificities of the two fungal enzymes and selected enzymes from Biocatalytics are compared in Table 2. These data suggest that in terms of substrate specificity both nitrilases examined herein are different

Table 1
Enzymatic hydrolysis of amino nitriles to non-proteinogenic amino acids

Substrate	Time (h)	*Fusarium solani* O1				*Aspergillus niger* K10			
		% nitrile	*ee*% nitrile	% acid	*ee*% acid	% nitrile	*ee*% nitrile	% acid	*ee*% acid
(±)-**1a**	18	73	20	27	56	64	10	36	14
(±)-**3a**	18	64	n.d.[a]	36	n.d.[a]	93	–	7	–
(±)-**4a**	18	90	–	10	–	100		0	
(±)-**5a**	18	0		100		0		100	
(±)-**5a**	2	17	53	83	11	14	57	86	9
(±)-**6a**	18	29	n.d.[a]	71	n.d.[a]	47	n.d.[a]	53	n.d.[a]
(±)-**7a**	18	91	–	9	–	98	–	2	–
(±)-**9a**	18	1[b]	3	96[b]	1	0		86[b,c]	15
(±)-**10a**	18	16	>99	84	19	0		98[b]	–
(±)-**10a**	2	60	37	40	55	43[b]	4	52[b]	3
(±)-**12a**	18	97	–	3	–	100		0	
(±)-**13a**	18	97	–	3	–	93	–	7	–
14a	18	3		97		2[b]		93[b]	
15a	18	4		96		1[b]		98[b]	

[a] Due to overlaid peaks *ee* determination was not possible.
[b] The respective amides were detected in the amount of the complementing percentage to 100%.
[c] *ee* of amide: 91%.