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Section: Microbial and Enzyme Technology

Genome mining for the discovery of new nitrilases in filamentous fungi

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Abstract

Purpose of work. Our aim is to describe new fungal nitrilases whose sequences were published but whose catalytic properties were unknown. We adapted for expression in *E. coli* three of the genes and confirmed that the enzymes acted on organic nitriles.

The genome mining approach was used to search for nitrilases in filamentous fungi. Synthetic genes encoding nitrilases in *Aspergillus niger*, *Gibberella moniliformis* and *Neurospora crassa* were expressed in *Escherichia coli*. This is the first heterologous expression of fungal enzymes of this type. The recombinant enzyme derived from *G. moniliformis* was an aromatic nitrilase with an activity of 390 U l⁻¹ culture with benzonitrile as substrate. This was much less than the activities of the recombinant enzymes derived from *A. niger* and *N. crassa* that had activities of 2500 and 2700 U l⁻¹ culture, respectively, with phenylacetone nitrile as substrate.

Keywords: Aromatic nitrilase · Arylacetonitrilase · *Aspergillus niger* · *Gibberella moniliformis* · *Neurospora crassa*

Introduction

Nitrilases are useful for the preparation of carboxylic acids or amides from nitriles. However, their selectivity or stability is often a limitation to their applicability (Zhu et al. 2008). New nitrilases (wild-type or mutant enzymes) with improved properties would therefore be advantageous.

Most nitrilases have been obtained by either strain enrichment (Asano et al. 2002) or by a metagenomic approach (Robertson et al. 2004). Genome mining, though, has become popular and new nitrilases have been obtained in this way from *Synechocystis* sp. (Heinemann et al. 2003), *Pyrococcus abyssi* (Mueller et al. 2006), *Bradyrhizobium japonicum* (Zhu et al. 2008; Seffernick et al. 2009) and *Burkholderia xenovorans* (Seffernick et al. 2009). Several cyanide hydratases from fungi were also obtained using this method (Basile et al. 2008). Here we have used this approach to search for new nitrilases in fungi which appears to be an abundant but so far relatively untapped, source of these enzymes (Kato et al. 2000; Martínková et al. 2009).

Materials and methods

Database mining and sequence alignment

Database searches were performed using the BLASTX and BLASTP programmes (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>) and the multiple alignment of amino acid sequences using COBALT: Constraint-based Multiple Alignment Tool (<http://www.ncbi.nlm.nih.gov/tools/cobalt/cobalt.cgi>).

DNA manipulations

Synthetic *nit* genes (GeneArt, Regensburg, Germany; Table 1) were ligated into the *Nde*I and *Hind*III sites in pET 30a(+) plasmid (Novagen). The codon frequency was optimized using GeneArt's own software. The resulting vectors were transformed into *E. coli* BL21-Gold (DE3) (Agilent Technologies – Stratagene Products) to give strains OK103 through OK105 (Table 1).

Enzyme assay

Nitrilase activity was assayed with an appropriate amount of whole cells in Tris/HCl buffer (50 mM, pH 8.0; 150 mM NaCl) with benzonitrile or phenylacetoneitrile (25 mM each) as substrate. After 10 min at 30 °C with shaking, concentrations of substrates and products were determined by HPLC.

Results and discussion

Selection and heterologous expression of genes encoding hypothetical nitrilases

To obtain new nitrilases from filamentous fungi, GenBank was searched for *nit* genes and three distantly related ones (with amino acid identities of approximately 40 %; see Online resource 1 for multiple sequence alignment) were selected. These genes encoded hypothetical nitrilases in *Gibberella moniliformis* (GenBank: ABF83489), *Aspergillus niger* CBS 513.88 (GenBank: XP_001397369) and *Neurospora crassa* OR74A (GenBank: CAD70472).

Genes optimized in terms of codon bias in *Escherichia coli* were synthesized (see Online resource 2 for optimized gene sequences), cloned into the pET 30a(+) plasmid and expressed under the T7 promoter without the His-Tag. After nitrilase induction with IPTG, all recombinant strains expressed protein subunits with the expected molecular weights (36-37 kDa). SDS-PAGE indicated nitrilase expression levels increasing in the following order: *G. moniliformis* << *A. niger* < *N. crassa* (Fig. 1).

Fig. 1

The nitrilase activities were assayed with benzonitrile and phenylacetoneitrile as typical substrates of aromatic nitrilases and arylacetoneitrilases, respectively (O'Reilly and Turner 2003). Although the expression protocol was not optimized, the nitrilase activity yields in all recombinant strains were significant (see Table 1). The nitrilase from *G. moniliformis* exhibited a high preference for benzonitrile, but the activities of the other two enzymes studied here were two orders of magnitude higher for phenylacetoneitrile than for benzonitrile.

Table 1

Aromatic nitrilase from *Gibberella moniliformis*

The strain expressing the *nit* gene from *G. moniliformis* had approx. 10 times higher activity with benzonitrile than with phenylacetoneitrile. Therefore, according to the classification work of Kobayashi and Shimizu (1994), the enzyme from this fungus was designated an aromatic nitrilase.

According to a mass spectrometric analysis of the tryptic fragments of two nitrilases from *Fusarium solani* strains IMI16840 and O1, these enzymes exhibited an amino acid sequence of high homology to the nitrilase from *G. moniliformis* (Vejvoda et al. 2010). These nitrilases from the *Fusarium* genus were also aromatic nitrilases. Their yields in the wild type producers of *F. solani* (1500 – 3000 U l⁻¹ with benzonitrile; Vevoda et al. 2008, 2010) were higher compared to *E. coli* expressing the *nit* gene from *G. moniliformis* (Table 1). However, production of the nitrilases in *F. solani* has been optimized by using a two-stage cultivation. Thus the heterologous production of the presumably highly similar enzyme from *G. moniliformis* was less laborious and required shorter cultivation times (20 h and 68-72 h for the heterologous and wild type strain, respectively).

A distantly related aromatic nitrilase from *Aspergillus niger* K10 was expressed in the wild type strain with a yield of approx. 100 U l⁻¹ (Kaplan et al. 2006), which was increased to approx. 500 U l⁻¹ by using an optimized heterologous expression protocol (IPTG concentration, time of induction, temperature shift after induction *etc.*; O. Kaplan, unpublished data). It is reasonable to expect that modifying these parameters will also be helpful in optimizing the expression of the *G. moniliformis* recombinant nitrilase.

Arylacetonitrilases from *Aspergillus niger* and *Neurospora crassa*

The activity yields of recombinant nitrilases from *A. niger* and *N. crassa* (over 2500 U l⁻¹ for phenylacetoneitrile) were in accordance with their high expression levels. The activities of these enzymes for benzonitrile were approx. 100 times lower. Members of two nitrilase subclasses - arylacetoneitrilases and aliphatic nitrilases - readily hydrolyze phenylacetoneitrile (O'Reilly and Turner 2003) but the latter, such as the aliphatic nitrilase from *Rhodococcus rhodochromus* K22 (Kobayashi et al. 1990), also hydrolyzed benzonitrile at comparable rates. Thus the new fungal enzymes appear to

be arylacetonitrilases which have so far only been reported in bacteria (Thuku et al. 2009) but, as far as we know, not in filamentous fungi.

Conclusion

We confirmed that the *E. coli* expression system used by ourselves is suitable for a number of recombinant nitrilases from filamentous fungi. It enabled nitrilases from the genera *Gibberella* (aromatic nitrilase) and *Neurospora* (arylacetonitrilase) to be expressed for the first time. Another new enzyme of the latter type was found in the *Aspergillus* genus, which seems to be an abundant source of nitrilases with varying substrate specificities. The ongoing purification and characterization of these enzymes will indicate the areas where they can potentially be used as biocatalysts.

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Table 1 Nitrilase activity of recombinant *Escherichia coli* strains expressing *nit* genes

Strain	Gene source	Total nitrilase activity (U L ⁻¹ culture)
OK103	<i>Neurospora crassa</i>	2678 ± 582 (phenylacetone nitrile) ^a
OK104	<i>Gibberella moniliformis</i>	390 ± 22 (benzoinitrile) ^b
OK105	<i>Aspergillus niger</i>	2512 ± 217 (phenylacetone nitrile) ^a

^ameans of three independent experiments

^bmeans of two independent experiments

E. coli BL21-Gold (DE3) strains OK103 through OK105 were grown in 50-ml Falcon tubes with 10 ml of LB broth with 50 µg kanamycin ml⁻¹. After 3.5 h of cultivation at 25 °C, the expression of nitrilase was induced with 0.5 mM IPTG. Cells were harvested after a further 17 h and assayed for

nitrilase activity with benzonitrile or phenylacetonitrile. Activities for preferential substrates are shown.

Fig. 1 Cell-free extracts obtained by cell sonication and removal of cell debris at 13,000 g and 4 °C were analyzed by 12 % SDS-PAGE followed by Coomassie staining. (1) *N. crassa*, (2) *G. moniliformis* and (3) *A. niger*. U = uninduced; I = induced; Nit = nitrilase