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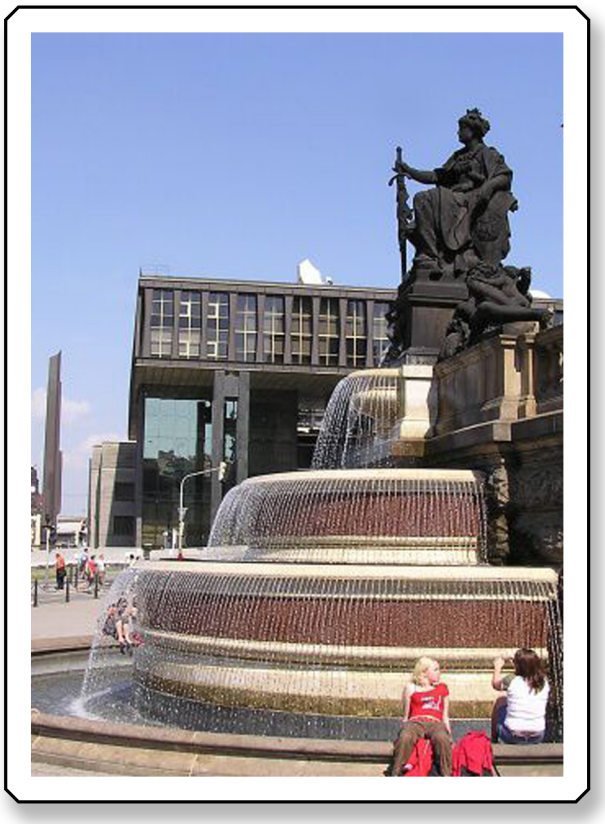

May 16, 2009 - Prague

Meeting of the WG 3

Cascade reactions on the nitrile group

Meeting venue:
Institute of Microbiology,
Academy of Sciences of the Czech Republic,
Vídeňská 1083, 142 20 Praha 4
Building X_A

www.biomed.cas.cz/mbu/en

Local organizer:
Dr. Ludmila Martínková
email: martinko@biomed.cas.cz
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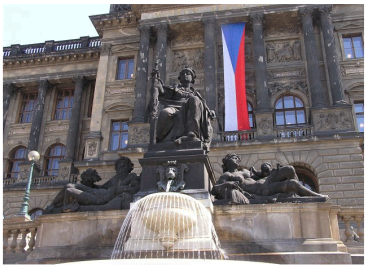
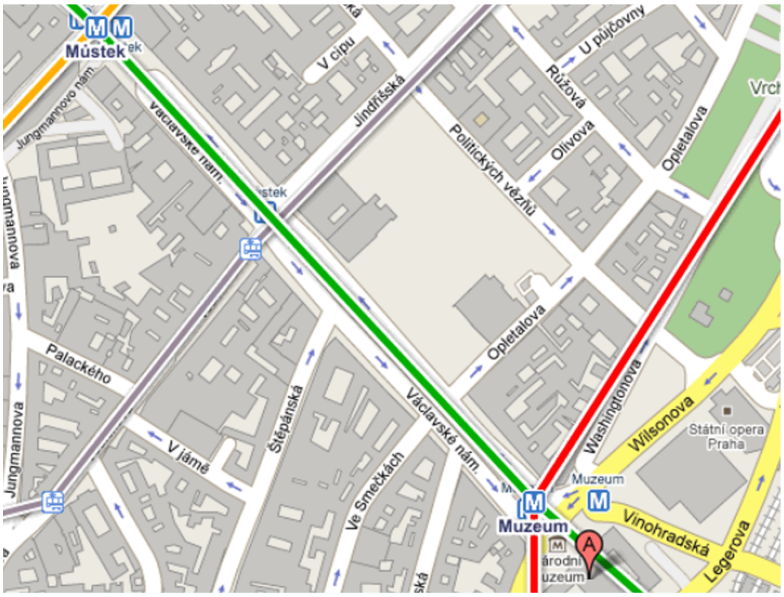
THE FRONT PAGE FOUNTAIN

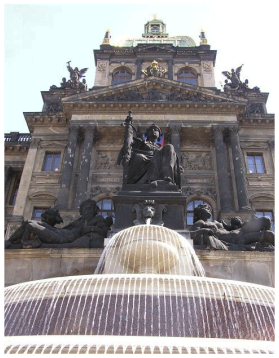
The fountain is located at the top of „Václavské náměstí“ (Wenceslas Square) in Prague. It is situated in front of the National Museum. It was created in 1889-91 by the sculptors Pavel Wagner and Čeněk Vosmík, and there are several other beautiful sculptures in the surroundings. The fountain was originally made of sandstone (originating from north Bohemia), red granite (originating from Corsica) and trout marble (originating from Austria). The Corsica red granite was later replaced by Scandinavian red granite during the reconstruction in 1995. Bronze waterspout poses cartouche of a lion. Its author is sculptor Bohuslav Schnirch, and it was bronze-casted in 1890 by Václav Mašek. Water descends over arches of the cascade. During the construction of Prague metro system the fountain was disassembled for a certain period of time. Since then it is under the supervision of the National Museum.

Public transport connections:

- metro lines A and C, station “Muzeum”
- tram line 11, station “Muzeum“







SATURDAY, 16 MAY 2009

9.15 - 9.25 **Vladimír Křen** (*Head of the Department of Biogenesis and Biotechnology of Natural Compounds, Inst. Microbiol., Prague, CZ*)
"WELCOME ADDRESS"

9.25 - 9.30 **Ludmila Martínková** (Inst. Microbiol., Prague, CZ)
"ORGANIZATION REMARKS"

Session I.....Chair: Laura Cantarella (Univ. Cassino, IT)

9.30 - 10.00 **Margit Winkler** (TU Graz - IMBT, AT)
"NITRILASE VARIANT WITH ENHANCED STABILITY AT ACIDIC CONDITIONS"

10.00 - 10.30 **Giovanni Gambera** (CNR Catania, IT)
"PERSPECTIVES IN THE SYNTHESIS OF NOVEL BIOMEDICAL VALUED NITRILE DERIVATIVES AND THEIR BIOTRANSFORMATIONS"

10.30 - 11.15 *Coffee break*

Session II..... Chair: Linda Otten (TU Delft, NL)

11.15 - 11.45 **Karel Bezouška** (Invited expert; Charles University, Prague, CZ)
"FOLDING OF FUNCTIONALLY ACTIVE PROTEINS IN VITRO: NEW POSSIBILITIES AND NEW PROCEDURES"

11.45 - 12.15 **Olga Sosedov** (Univ. Stuttgart, DE)
"AMIDE SYNTHESIS VIA NITRILASE FROM PSEUDOMONAS FLUORESCENS EBC 191"

12.15 - 12.45 **Ondřej Kaplan** (Inst. Microbiol., Prague, CZ)
"HETEROLOGOUS EXPRESSION OF FUNGAL NITRILASES"

12.45 - 14.00 *Lunch*

Session III..... Chair: Nicola D´Antona (CNR Catania, IT)

14.00 - 14.30 **Miroslav Pátek** (Inst. Microbiol., Prague, CZ)
"ANALYSIS OF NITRILE HYDRATASE-AMIDASE OPERON FROM RHODOCOCCUS ERYTHROPOLIS"

14.30 - 15.00 **Maria Cantarella** (Univ. L´Aquila, IT)
"USE OF NITRILE HYDRATASE-AMIDASE CASCADE SYSTEM FOR NITRILE BIOCONVERSION"

15.00 - 15.30 **Sander van Pelt** (TU Delft, NL)
"NITRILE HYDRATASES IN ORGANIC SYNTHESIS"

15.30 - 16.00 **Linda G. Otten** (TU Delft, NL)
"CLONING AND EXPRESSION OF NEW NITRILE HYDRATASES"

16.00 - 16.45 *Coffee break; Discussions in groups*

Transfer to working dinner (by city transport and a short walk round the Vyšehrad castle)

18.00 *Working dinner*
(Restaurant "U Šemíka", Vratislavova 36, near Vyšehrad; http://www.usemika.cz)

NITRILASE VARIANT WITH ENHANCED STABILITY AT ACIDIC CONDITIONS

Ulrike Schreiner, Margit Winkler

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Nitrilases catalyze the hydrolysis of nitriles to their corresponding carboxylic acids and ammonia. They exhibit their optimal activity at neutral pH values. For hydrolysis of base labile substrates such as cyanohydrins we aspired to improve the stability of the *Alcaligenes faecalis* nitrilase (NIT*Af*, swissprot: P20960) for the hydrolysis of cyanohydrins at low pH by directed evolution. Mutant pHNIT45 showed a 3 to 4 fold higher specific activity compared to NIT*Af* upon incubation at pH 4.5 for 30 minutes. To prove the applicability of the newly engineered pHNIT45 we studied the conversion of (R)-2-Cl-mandelonitrile to (R)-2-Cl-mandelic acid at pH 4.5 and pH 7.5 and compared it to NIT*Af.* At pH 4.5 (R)-2-Cl-mandelic acid was obtained with improved conversion and higher enantiopurity than NIT*Af* at pH 7.5.

PERSPECTIVES IN THE SYNTHESIS OF NOVEL BIOMEDICAL VALUED NITRILE DERIVATIVES AND THEIR BIOTRANSFORMATIONS

Nicola D'Antona[1], Giovanni Gambera[1], Ondřej Kaplan[2], Ludmila Martínková[2], Giovanni Nicolosi[1], Giuseppe Salvo[3]

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3) Istituto Biochimico Italiano "Giovanni Lorenzini" SpA, V.Fossignano 2, 04011 Aprilia (LT), Italy

Biocatalysis represents today a well established field of research at a crossroad between organic synthesis and biotechnology, dealing with the application of biological systems such as microorganisms, enzymes or catalytic antibodies to the synthesis of organic compounds. The main advantage of biocatalysis is related to the fact that these systems, generally defined as biocatalysts, can be used in aqueous solutions under mild experimental conditions of temperature and pH, so allowing to operate in a "green manner" and to realise "sustainable" synthetic processes.

Because of their ability to catalyse the hydrolysis of nitriles to acids and/or amides, enzymes belonging to the superfamily of nitrilases have a great potential both in organic synthesis (especially when aimed to the preparation of bioactive compounds with specific and unique chemical-physical properties), and in the development of "bioremediation" tools for waste treatment processes. Moreover, the enzymatic hydrolysis of racemic nitriles into enantiomerically pure carboxylic acids is of great industrial interest. To date, very little is known about the use of these biocatalyst in the recognition of bulky rigid aliphatic nitrilic molecules, that by enantio- and regio-biotransformations could open amazing applications in the fields of organic synthesis, medicinal and pharmaceutical chemistry, nanobiotechnologies.

In this lecture examples and future perspectives concerning the chemo-enzymatic synthesis of novel nitrile-cyclitols useful as synthons in the preparation of biologically important molecules, will be presented. Examples of biotrasformations aimed to the enantio-preparation of non steroidal anti-inflammatory drugs belonging to the profen family will be illustrated too.

Finally, hypothesis in the synthesis and biotrasformations of potentially valuable molecules with different and more complex chirality than the central one, will be discussed.

FOLDING OF FUNCTIONALLY ACTIVE PROTEINS *IN VITRO*: NEW POSSIBILITIES AND NEW PROCEDURES

Karel Bezouška[1,2], Ondřej Kaplan[2], Alena Hájková[1], Kristýna Kotýnková[1], Pavel Hanč[1], Oldřich Benada[2], Ludmila Martínková[2]

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An information code behind the protein folding constitutes one of the most important and most challenging questions of contemporary bioscience. Protein folding is a highly cooperative process in which the presence of key evolutionarily conserved amino acid residues within the protein's primary structure remains critical (1). However, until today the detailed knowledge of the protein folding "pathways" are available for only a few proteins, and the biotechnological task of folding of proteins into functionally active native states thus remains a matter of empirical trials and errors, as well as one of the bottlenecks in the current large scale structural biology projects (2). The talk is going to cover new developments in this area from the standpoint of the use of bioinformatic protein folding databases, high throughput protein folding kits, development of new chemistries and new additives useful in protein folding, and development of rapid and convenient techniques to monitor the protein folding status (3). The lecture will be supplemented by several recent examples illustrating the use of these new possibilities and new protein folding procedures to achieve folding of those proteins that are difficult to refold using the traditional methodology.

(1) Dill KA et al (1995) *Protein Sci* **4,** 561.
(2) Smock RG et al (2005) *Cell* 122, 832.
(3) Fulton R. et al (2007) *Nucleic Acid Res* **35,** D304.

Supported by grants from Ministry of Education of the Czech Republic (MSM_21620808 and 1M0505), and from Grant Agency of the Academy of Science of the Czech Republic (IAA500200708).

CONSTRUCTION OF THE NITRILASE VARIANTS FROM *PSEUDOMONAS FLUORESCENS* EBC 191 WITH INCREASED AMIDE FORMATION CAPACITY

Olga Sosedov, Andreas Stolz

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The bacterium *P. fluorescens* EBC 191 produces an arylacetonitrilase, which converts many α-substituted nitriles to the corresponding carboxylic acids and different amounts of the corresponding amides. We are currently attempting to exploit this "nitrile hydratase activity" for the synthesis of hydroxycarboxamides from aldehydes or ketones and a cyanide source. The wild-type nitrilase converted the model substrate (*R*,*S*)-mandelonitrile to mandelic acid and mandelic acid amide in a ratio of about 6:1. In previous work it was found that different C-terminally truncated nitrilase mutants and nitrilase variants with amino acid changes in the vicinity of the catalytical center produced almost equimolar amounts of mandelic acid and mandelic acid amide [1]. In the course of the present work it was tried to further increase the amide formation. Therefore, a nitrilase variant was constructed which combined a C-terminal deletion of 60 amino acids together with an exchange of Cys163 against an asparagine residue. Indeed a cumulative effect was observed and 70% of mandelic acid amide was formed from racemic mandelonitrile.

In a second approach random mutagenesis experiments were performed using error-prone PCR in order to improve the nitrile hydratase activity of the enzyme. The nitrile hydratase activities of the recombinant clones obtained were determined *in-vivo* by using a recombinant *E. coli* strain synthesising a bacterial amidase from *Rhodococcus erythropolis* MP50. The individual mutated variants of the *P. fluorescens* nitrilase were expressed simultaneously with the amidase. The growing colonies were incubated with a solution of mandelonitrile plus hydroxylamine. In this system the amidase transferred the acyl group from the produced amide to hydroxylamine to give the corresponding hydroxamic acid. The subsequent addition of Fe^{3+}-ions resulted in the formation of deep red Fe^{3+}-hydroxamic acid complexes [2]. It was demonstrated that a more intensive staining correlated with a higher level of nitrile hydratase activity in the tested nitrilase mutants.

Currently from two mutant libraries about 500 mutants were tested and 4 nitrilase variants identified which produced significantly increased amounts of mandelic amide from mandelonitrile. The encoding genes were sequenced and found to contain 3-6 amino acid changes per gene.

[1] Kiziak *et al.* (2007) Protein Eng. Des. Sel. 20:385-396
[2] Reisinger *et al.* (2003) EP 1382689A1

HETEROLOGOUS EXPRESSION OF FUNGAL NITRILASES

Ondřej Kaplan[1], Ludmila Martínková[1], Karel Bezouška[1,2]

1) Institute of Microbiology, Academy of Science of the Czech Republic, Vídeňská 1083, 14220 Praha 4; ondrej.kaplan@gmail.com
2) Department of Biochemistry, Faculty of Science, Charles University Prague, Hlavova 8, 12840 Praha 2

Five fungal nitrilases were expressed in *E. coli* BL21 Gold (DE3) using synthetic genes (GeneArt). The sequences encoding putative nitrilases were optimized for expression in *E. coli* and ligated into expression vector pET-28a(+) or pET-30a(+). Target protein showing nitrilase activity towards benzonitrile was expressed in case of all five synthetic genes. Activity of the nitrilases from *Gibberella moniliformis* and *Penicillium marneffei* ATCC 18224 was significantly improved by co-expression with bacterial chaperone teams (see Fig. 1). The nitrilase from *G. moniliformis* was partially purified by ionex chromatography and gel filtration. The nitrilase from *P. marneffei* bearing a 6His-tag was purified by metal affinity chromatography. Both nitrilases were copurified with a 60 kDa protein, probably the bacterial chaperonin GroEL. Recombinant nitrilase from *G. moniliformis* is the first fungal nitrilase expressed in *E. coli* with high specific activity for benzonitrile – approx. 91 U/mg of protein (assayed at pH 8.0 and 40 °C).

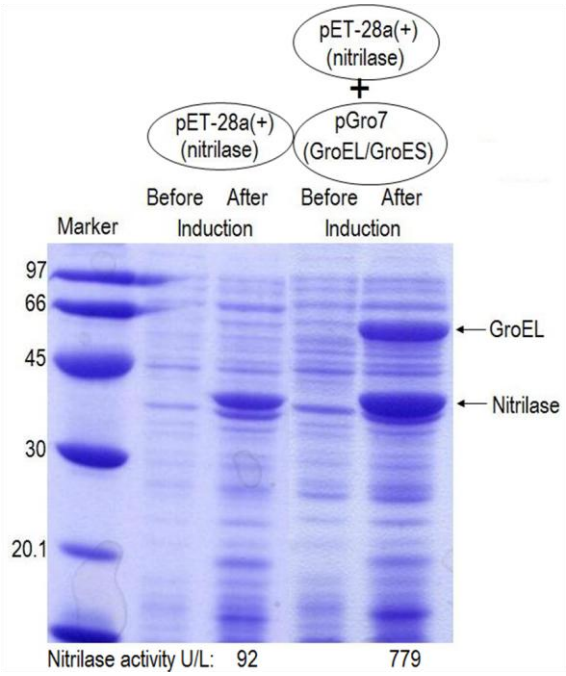

Fig 1: SDS-PAGE of cell extracts from *E. coli* cells harbouring the gene of the putative nitrilase from *Penicillium marneffei* ATCC18224 cloned into pET-28a(+). Expression without or with bacterial chaperones GroEL/GroES cloned into chaperone plasmid pGro7.

Supported by Grant Agency of the ASCR (IAA500200708) and by grants from Ministry of Education of the Czech Republic (LC06010, OC09046).

ANALYSIS OF NITRILE HYDRATASE-AMIDASE OPERON FROM *RHODOCOCCUS ERYTHROPOLIS*

Miroslav Pátek, Adam Pavlík, David Kubáč, Jan Nešvera

Institute of Microbiology, Academy of Sciences of the Czech Republic, Laboratory of Molecular Genetics of Bacteria, Vídeňská 1083, CZ-142 20 Praha 4, Czech Republic; patek@biomed.cas.cz

Rhodococcus erythropolis A4 is an efficient producer of the Fe-type nitrile hydratase and amidase which were used for a hydrolysis of (aryl)aliphatic, (hetero)aromatic and alicyclic nitriles and amides, respectively. To examine the chromosomal organization of the respective genes, we have cloned the genes *ami* (amidase) and *nha1-nha2* (α- and β-subunits of nitrile hydratase) from *R. erythropolis* A4. The nitrile hydratase-amidase genes were found to be linked and form together with *oxd* (aldoxime dehydratase) and *nhr1, nhr2, nhr3* and *nhr4* coding for regulatory proteins an operon-like structure

nhr4→ oxd→nhr2→nhr1→ ami→ nha1→ nha2→ nhr3→

Analysis of the complete nucleotide sequence of the cluster (9552 bp, GenBank Acc. No. AM946017) revealed high homology with the nitrile hydratase gene cluster from *R. erythropolis* PR4 (GenBank Acc. No. AP008957) and *R. globerulus* A-4 (GenBank Acc. No. AB105912). The *nhr4* gene codes for an AraC-type transcriptional regulator, which may be involved in expression of the cluster. The *nhr3* gene codes for a nitrile hydratase activator that is probably involved in correct folding of the protein and/or incorporation of Fe into nitrile hydratase. The functions of *nhr1* and *nhr2* are unknown. The *nhr2* gene was deleted, but no effect on nitrile hydratase activity was observed. The upstream regions of the individual genes were cloned in the promoter-probe vector pEPR1 and their activity in *R. erythropolis* was analyzed using the fluorescence of the GFP (green fluorescent protein) reporter. Promoter of the *ami* gene was precisely localized by determination of the *ami* transcription start point. The *ami* gene is transcribed in a separate transcript (1.6 kb), in a common transcript with *nha1* and *nha2* (3 kb) and in another transcript covering *ami, nha1, nha2* and *nhr3* (5 kb), according to Northern analysis. The *ami* gene was cloned in the expression vectors pEXT20 (replicating in *E. coli*) and pFEX16 (replicating in *R. erythropolis*). Activity of amidase produced by *E. coli* cells confirmed that no additional gene is necessary for its expression.

Supported by grant LC06010 from Ministry of Education of the Czech Republic.

USE OF NITRILE HYDRATASE-AMIDASE CASCADE SYSTEM FOR NITRILE BIOCONVERSION

Maria Cantarella[1], Laura Cantarella[2], Alberto Gallifuoco[1], Anna Malandra[1], Agatha Spera[1], Ludmila Martínková[3]

1) *Department of Chemistry, Chemical Engineering and Materials, University of L'Aquila, Monteluco di Roio, 67040, L'Aquila, Italy, maria.cantarella @univaq.it*
2) *Department of Industrial Engineering, University of Cassino, via di Biasio 43, 03043 Cassino (FR), Italy,*
3) *Institute of Microbiology, Academy of Sciences of the Czech Republic, 142 20 Praha, Czech Republic*

Microbacterium imperiale CBS 498-74 resting cells has been used as a catalyst for the bioconversion of some nitriles into the corresponding acid. This strain follows a two-step nitrile degradation pathway; the first reaction, catalysed by nitrile hydratase (NHase), promotes the formation of an amide, as intermediate, which is further transformed into the corresponding acid in a second reaction catalyzed by amidase (AMase).

The reactions were investigated in both batch and continuous stirred membrane bioreactors (CSMR). This reactor configuration allows the independent kinetic characterisation of each enzyme in the resting cell by feeding the reactor with the appropriate substrate: the nitrile or the amide. In the years, depending on the substrate investigated high reaction conversion were obtained 000. The bioconversion of acrylonitrile and propionitrile into the corresponding amides was realized, as the appropriate choice of CSMR operational conditions made the AMase activity negligible as compared with NHase activity. Near theoretical 100% conversion yields into amide were reached.

The case study of 3-cyanopyridine bioconversion revealed a different temperature and substrate dependence of the enzymes. On one hand, the NHase, which is active at lower substrate concentration, was proved to be more labile and imposed low temperature (at most 10°C) in kinetic runs. On the other hand, the AMase activity, stable up to 50 °C, was partially inhibited by nicotinamide (substrate) at concentrations of $\geq$ 300 mM.

In the continuous stirred membrane bioreactors (CSMR) operational parameters such as temperature, cell load, residence time and substrate feeding strategy are key factors to investigate. Appropriate residence time, for instance, in a single CSMR allowed us to obtain high conversion of nicotinamide (up to 88%) [4]. A further increase in substrate conversion was obtained operating the reaction in CSM-reactors arranged in series at conditions chosen to promote in the first one the NHase catalysis and in the second one that of AMase.

[1] Cantarella M., Cantarella L., Spera A., Alfani F. "Acrylamide production in an ultrafiltration-membrane bioreactor using cells of *Brevibacterium imperialis* CBS 489-74" J Membr Sci 147 (1998) 279-290
[2] Cantarella M., Cantarella L., Gallifuoco A., Frezzini R., Spera A., Alfani F. "A study in UF-membrane reactor on activity and stability of nitrile hydratase from *Microbacterium imperiale* CBS 498-74 resting cells for propioamide production" J Mol Catal B-Enzym 29 (2004)105-113
[3] Cantarella M., Cantarella L., Gallifuoco A., Spera A. "Use of a UF-membrane reactor for controlling selectively the nitrile hydratase - amidase system in *Microbacterium imperiale* CBS 498-74 resting cells. Case study: benzonitrile conversion". Enzyme Microb Technol 38 (2006) 126-134
[4] Cantarella M, Cantarella L, Gallifuoco A, Intellini R, Kaplan O, Spera A, Martínková L, Amidase-catalyzed production of nicotinic acid in batch and continuous stirred membrane reactors, Enzyme Microb Technol 42 (2008) 222-229

Supported by University of L'Aquila Research Fund, COST CM0701 and grant OC09046 from Ministry of Education of the Czech Republic

NITRILE HYDRATASES IN ORGANIC SYNTHESIS

Sander van Pelt, Linda G. Otten, Fred van Rantwijk, Roger A. Sheldon

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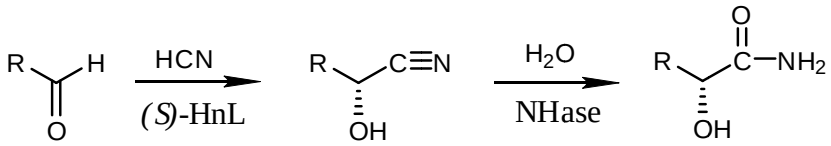
Nitrile hydratases (NHases, E.C. 4.2.1.84) catalyse the transformation of nitriles into the corresponding amides and were first discovered 25 years ago in studies on the microbial degradation of toxic cyano-group-containing compounds [1]. The use of NHases in synthetic chemistry is especially interesting in conversions where the substrate or the product is labile at the high temperature or extreme pH employed in chemical nitrile hydration. However, also in the production of bulk chemicals like acrylamide, NHases offer a green alternative to chemical hydration. Besides mild reaction conditions, the potential advantage of using a nitrile hydratase as a catalyst is a high chemo-, regio-, and enantioselectivity [2].

The present limited application of NHases in organic synthesis is caused by the scarcity of commercially available well-characterised NHases, as well as by their low stability in cell-free preparation and generally low enantioselectivity. In our presentation we demonstrate that it was possible to significantly increase the stability of a cell-free NHase from a new nitrile degrading haloalkaliphilic bacterium [3] by immobilising this enzyme in the form of a cross-linked enzyme aggregate (CLEA) [4]. The drawback of the low NHase enantioselectivity was addressed by combining the aforementioned NHase CLEA with a CLEA of the hydroxynitrile lyase (HnL, E.C. 4.1.2.37) from *Manihot esculenta*. The combination of these enzymes enabled us to perform a one-pot bienzymatic cascade for the production of enantiomerically enriched (ee = 84 -90 %) aliphatic α-hydroxycarboxylic amides from the corresponding aldehydes and HCN.



Scheme 1: Bienzymatic one-pot cascade using an immobilised HnL and NHase for the production of aliphatic *(S)*-α-hydroxycarboxylic amides

Finally, a small library of five purified NHases was screened on enantioselectivity for several structurally different nitriles. Some of these NHases showed promising enantioselectivities and these results will be discussed.

[1] Y. Asano, Y. Tani, H. Yamada, *Agric. Biol. Chem.* **1980**, *44*, 2251.
[2] M-X. Wang, *Top. in Cat.* **2005**, *35*, 117
[3] D. Y. Sorokin, S. van Pelt, T. P. Tourova, G. Muyzer, *Appl. Environ. Microbiol.* **2007**, *73*, 5574
[4] S. van Pelt, S. Quignard, D. Kubač, D. Yu. Sorokin, F. van Rantwijk, R. A. Sheldon, *Green Chem.* **2008**, *10*, 395

NEW NITRILE HYDRATASES FOR BIOCATALYSIS

Linda G. Otten, Sander van Pelt, Isabel Arends

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Nitrile hydratases (NHases, E.C. 4.2.1.84) were discovered 25 years ago in studies of microbial degradation of toxic cyano-group-containing compounds [1]. They can catalyse the transformation of nitriles into the corresponding amides (fig. 1). The use of NHase in synthetic chemistry only shows few industrial applications. At this moment, NHases offer a green alternative to chemical nitrile hydrolysis in the production of bulk chemicals like acrylamide or nicotinamide, but they are only used for non-stereoselective reactions [2].

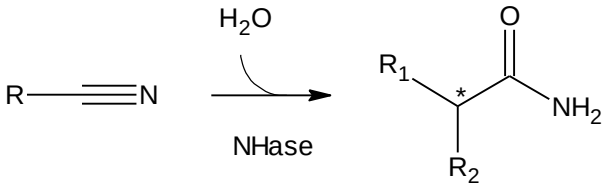

Fig. 1: Reaction catalysed by NHase

Enantioselectivity is an important aim in (bio)catalysis. Unfortunately not many enantioselective NHases have been described yet. In order to expand the availability of NHases, we enriched samples from soda soils and lakes on different nitriles and found a few organisms containing NHase activity [3]. The aim of our research is to study these NHases and use them as a biocatalyst. Therefore we are working on an expression system, which will allow us to produce and purify the enzyme. In order to pick the gene from these new and unknown organisms, we designed degenerate primers based on known NHases. The identification and isolation of these genes will be described.

[1] Asano, Y., Y. Tani & H. Yamada (1980). A new enzyme nitrile hydratase which degrades aetonitrile in combination with amidase. *Agric. Biol. Chem.* **44** (9): 2251-2252
[2] Van Pelt, S., F. van Rantwijk, R.A. Sheldon (2008). Nitrile hydratases in synthesis. *Chem. Today* **26** (3): 2-4
[3] Sorokin D.Y., S. van Pelt, T.P. Tourova, G. Muyzer (2007). Microbial isobutyronitrile utilization under haloalkaline conditions. *Appl. Environ. Microbiol.* **73** (17): 5574-5579

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