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Enantioselective nitrile hydratases and amidases from different bacterial isolates

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Abstract

Different enrichment cultures were performed using various nitriles as sole source of nitrogen, succinate or a mixture of sugars as carbon sources and soil samples as inocula. It was attempted to obtain a relationship between the selective nitrogen source and the enzyme systems which were synthesized by the isolates, the enzyme specificities, and enzyme enantioselectivities. Various strains were obtained which harbored enantioselective nitrilases, nitrile-hydratases or amidases. The enantioselective amidase from the isolate *Rhodococcus erythropolis* MP50 and the enantioselective nitrile hydratase from *Agrobacterium tumefaciens* d3 were studied in greater detail. The purified amidase from *R. erythropolis* MP50 hydrolysed 2-phenylpropionamide, naproxen amide and ketoprofen amide with enantiomeric excesses (ee) > 99% up to 49% conversion of the respective substrates. In the presence of hydroxylamine, the amidase also formed the corresponding hydroxamates enantioselectively. These chiral hydroxamates could be chemically converted by a Lossen rearrangement into chiral amines. The partially purified nitrile hydratase from *A. tumefaciens* d3 converted 2-phenylbutyronitrile and ketoprofen nitrile to the corresponding *S*-amides with ee values > 90% at 30% conversion of the respective substrate.

Keywords: Enantioselectivity; Hydroxamates; *Agrobacterium tumefaciens*; *Rhodicoccus erythropolis*

1. Introduction

The microbial hydrolysis of nitriles proceeds via two different pathways. Nitrilases participate in the direct conversion of nitriles to their corresponding carboxylic acids and ammonia. The alternative pathway involves nitrile hydratases which mediate the conversion of nitriles to the corresponding amides. In a subsequent step amidases convert the amides to the corresponding carboxylic acids and ammonia. The enzymatic hydrolysis of nitriles represents a very convenient synthetic method for amides/and or carboxylic acids due to the mild reaction conditions [1,2]. Furthermore, these enzymatic reactions also allow the enantioselective synthesis of optically active amides and carboxylic acids from racemic precursors [3,4]. Optically active α-substituted carboxylic acids (e.g., amino acids, α-hydroxycarboxylic acids

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or 2-arylpropionic acids) have various biological functions and usually only one enantiomer is metabolically effective. We therefore attempted to obtain new nitrile converting enzyme systems from nature with the ability to convert racemic nitriles to optical active carboxylic acids.

2. Results

2.1. Enrichments with aromatic nitriles as sole source of nitrogen

Bacteria were enriched with different aromatic nitriles, such as 2-phenylpropionitrile, 2-phenylbutyronitrile, naproxen nitrile, benzonitrile, or naphthalenecarbonitrile (Fig. 1) as sole nitrogen source and succinate as sole source of carbon and energy. Certain nitriles showed only a very limited water solubility. In these enrichments, it was tested if the addition of different organic phases influenced the isolation of nitrile hydrolysing bacteria.

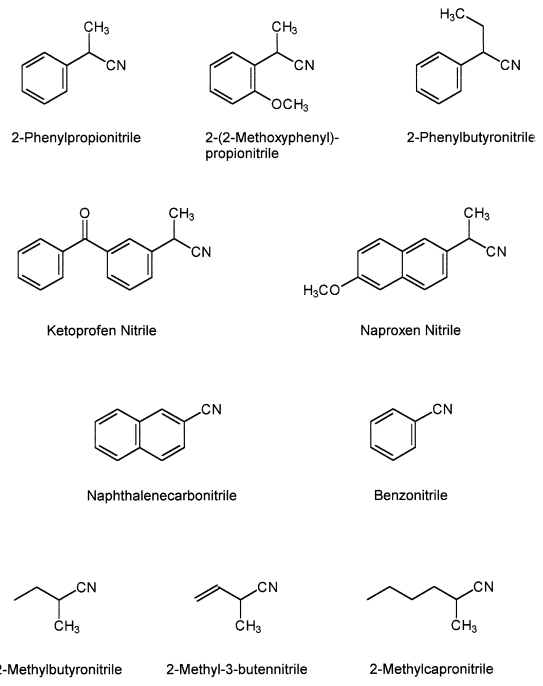


Fig. 1. Nitriles used as nitrogen sources during enrichments.

2-Phenylpropionitrile as nitrogen source resulted predominantely in the enrichment of Gram-negative bacteria which harboured nitrilase activity. With the other nitriles used, a substantial majority of Gram-positive strains mainly from the genus *Rhodococcus* were isolated. These strains contained predominantly a nitrile hydratase/amidase system. The conversion of different nitriles by the isolates was compared. The nitrile hydrolysing systems of the new isolates showed usually high activities against those nitriles which were used for the enrichment of the bacteria (Fig. 2) [5,6].

2.2. Enrichments with aliphatic nitriles

Different enrichments were performed with 2-methylbutyronitrile, 2-methyl-3-butenenitrile, 2-methylvaleronitrile or 2-methylcapronitrile (Fig. 1) as sole source of nitrogen and a mixture of glucose, fructose, and succinate as carbon source. From about 60 isolates, *Pseudomonas putida* IP08 showed the highest specific activities for the conversion of 2-methylbutyronitrile and was further analysed. Strain IP08 grew with 2-methylbutyronitrile and 2-methyl-3-butenenitrile as sole source of nitrogen, but did not utilize 2-methylvaleronitrile or 2-methylcapronitrile. The strain formed a nitrile-hydratase/amidase system, which converted 2-methylbutyronitrile rapidly to 2-methylbutyric acid amide and slowly hydrolysed the amide to the corresponding 2-methylbutyric acid [7].

2.3. Enantioselectivity of the amidases and nitrile hydratases from the isolates

Resting cell experiments with the isolates obtained from the enrichments with the aromatic subtrates suggested that the nitrilases and nitrile hydratases showed *R*- or *S*-selectivity with in general poor optical yields. In contrast,

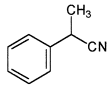
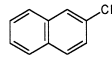
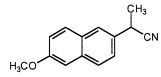
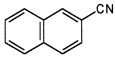
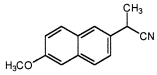
Compounds used for turnover experiments with resting cells	Relative activities of strains/ Enrichment substrates			
	Rhodococcus sp. BM5 	*Agrobacterium tumefaciens* d3 	Strain NM10 („*Alcaligenes* sp.“) 	*Rhodococcus erythropolis* MP50 
	100	810	15	not done
	40	100	10	400
	20	8	100	2700
	<1	0	4	100

Fig. 2. Relationship between the enrichment substrates and the substrate specificity of the nitrile hydrolysing enzyme systems from some new isolates.

the amidases were almost exclusively *S*-selective, often forming the optical pure acids (ee > 99%). The enantioselectivity was not dependent from a methyl-group in α-position to the nitrile or amide-group, because different isolates also converted α-aminophenylacetonitrile or α-aminophenylacetamide enantioselectively with ee values > 95% until more than 40% of the racemic substrates were converted (Figs. 3 and 4) [6,8].

2.4. Conversion of 2-methylbutyronitrile to R-2-methylbutyric acid

The conversion of 2-methylbutyronitrile by resting cells of *P. putida* IP08 to 2-methylbutyric acid amide and 2-methylbutyric acid also showed some enantioselectivity. The 2-methylbutyric acid amide which was formed quickly at the beginning of the reaction was almost racemic, but the slow turnover of the amide resulted intermediately in the formation of *R*-2-methylbutyric acid amide and *R*-2-methylbutyric acid. These results suggested the presence of a ±non-enantioselective nitrile-hydratase, a *S*-specific amidase and a *S*-specific

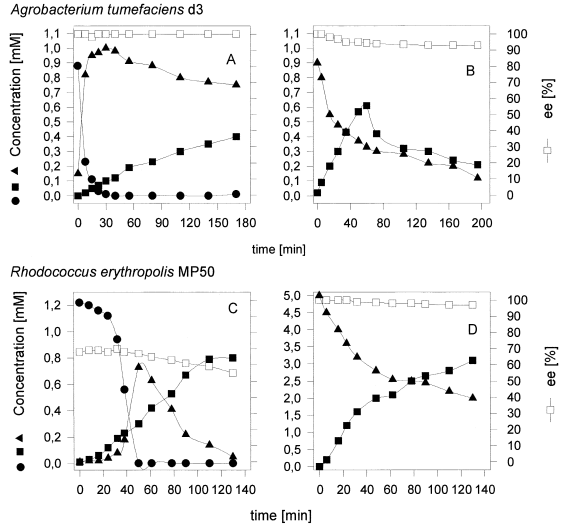


Fig. 3. Enantioselective formation of *S*-α-aminophenylacetic acid from racemic α-aminophenylacetonitrile (A,C) or racemic α-aminophenylacetamide (B,D) by resting cells of *Agrobacterium tumefaciens* d3 (A,B) or *Rhodococcus erythropolis* MP 50 (C,D). Resting cells (5 ml of cell suspension; 0.77–2.2 mg protein/ml with strain d3 and 0.1–0.43 mg protein/ml with strain MP 50) were incubated at 30°C in Erlenmeyer-flasks in a water bath shaker. The concentrations of α-aminophenylacetonitrile (●), α-aminophenylacetamide (▲), and α-aminophenylacetic acid (■) and the determination of the enantiomeric excess of *S*-α-aminophenylacetic acid were done by chiral HPLC.

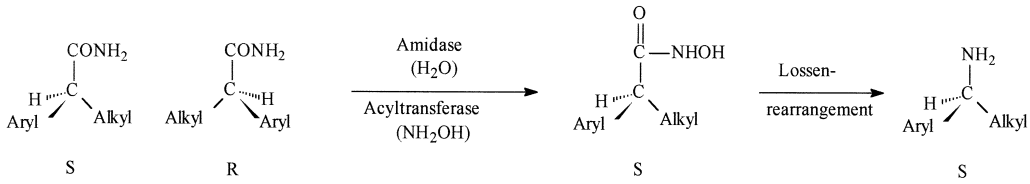


Fig. 4. Schematic diagram for the enantioselective formation of an optical active *S*-2-arylpropionhydroxamate from a racemic 2-arylpropionamide by the enantioselective amidase from *Rhodococcus erythropolis* MP50 and its subsequent chemical Lossen rearrangement to an optical active *S*-amine.

enzyme system which further converted *S*-2-methylbutyric acid [7].

2.5. The enantioselective amidase from R. erythropolis MP50

R. erythropolis MP 50 was isolated in the presence of pristane (50% v/v) with naproxen nitrile as nitrogen source. In contrast, no isolate was obtained from the same inoculum in the absence of an organic phase. The strain exhibited nitrile hydratase/amidase activity and converted naproxen nitrile to *S*-naproxen. The analysis of reaction showed that mainly the amidase was responsible for the enantioselectivity of the reaction [9]. Because an enantioselective amidase with high activity for naproxen amide has not been found before, the amidase was characterized and purified and the corresponding gene cloned and sequenced. The enantioselective amidase has a molecular weight of about 480,000 and is composed of identical subunits with a molecular weight of about 61,000. The NH_2-terminal amino acid sequence was significantly different from previously published sequences of bacterial amidases. The purified amidase hydrolysed a wide range of aliphatic and aromatic amides. The highest enzyme activities were found with amides carrying hydrophobic residues, such as pentyl or naphthoyl. The purified enzyme converted racemic 2-phenylpropionamide, naproxen amide [2-(6-methoxy-2-naphthyl)propionamide], and ketoprofen amide [2-(3′-benzoylphenyl)propionamide] to the corresponding *S*-acids with enantiomeric excess $> 99\%$ at almost 50% conversion of the racemic amides [10,11].

2.6. Formation of chiral hydroxamates and subsequent chemical Lossen-rearrangement to chiral amines

The amidase from *R. erythropolis* MP50 demonstrated in the presence of hydroxylamine acyltransferase activity and catalyzed the formation of hydroxamates from amides and hydroxylamine. The acyltransferase activity of the purified amidase for the substrates acetamide, phenylacetamide, and 2-phenylpropionamide were higher than the corresponding rates for the hydrolysis reactions. With the substrate 2-phenylpropionamide the hydrolysis reaction and the acyltransferase activity were highly enantioselective. The optical active 2-phenylpropionhydroxamate was converted by a chemical Lossen rearrangement in an aqueous medium into the enantiopure *S*-1-phenylethylamine [12,13].

2.7. Enantioselectivity of nitrile hydratases

The enrichments with 2-phenylpropionitrile as sole source of nitrogen resulted almost exclusively in the isolation of strains which contained nitrilase activity and were tentatively identified as *Pseudomonas* sp. The only exception was *A. tumefaciens* strain d3 which showed in resting cells experiments indications of an enantioselective nitrile hydratase [14]. Because only very limited information was available about the enantioselectivity of nitrile hydratases [3,15–17], the enzyme from *A. tumefaciens* d3 was purified and completely separated from the amidase activity which is also present in cell extracts

prepared from this strain. The enzyme hydrated various 2-arylpropionitriles and other aromatic and heterocyclic nitriles. With racemic 2-phenylpropionitrile, 2-phenylbutyronitrile, 2-(4-chlorophenyl)propionitrile, 2-(4-methoxy)propionitrile or ketoprofen nitrile the corresponding *S*-amides were formed enantioselectively. The highest enantiomeric excesses were observed with 2-phenylpropionitrile, 2-phenylbutyronitrile, and ketoprofen nitrile. For the amides formed from these compounds, ee values > 90% were observed until about 30% of the respective substrate were converted. For all substrates tested it was found that the *S*-enantiomers were preferentially hydrated [18].

References

[1] M. Kobayashi, S. Shimizu, FEMS Microbiol. Lett. 120 (1994) 217.
[2] H. Yamada, M. Kobayashi, Biosci. Biotech. Biochem. 60 (1996) 1391.
[3] H. Kakeya, N. Sakai, T. Sugai, H. Ohta, Tetrahedron Lett. 32 (1991) 1343.
[4] T. Beard, M.A. Cohen, J.S. Parratt, N.J. Turner, J. Crosby, J. Moilliet, Tetrahedron Asymmetry 4 (1993) 1085.
[5] N. Layh, A. Stolz, S. Förster, F. Effenberger, H.-J. Knackmuss, Arch. Microbiol. 158 (1992) 405.
[6] N. Layh, B. Hirrlinger, A. Stolz, H.-J. Knackmuss, Appl. Microbiol. Biotechnol. 47 (1997) 668.
[7] M. Binder, PhD thesis, Universität Stuttgart, 1997.
[8] B. Hirrlinger, PhD thesis, Universität Stuttgart, 1996.
[9] N. Layh, A. Stolz, J. Böhme, F. Effenberger, H.-J. Knackmuss, J. Biotechnol. 33 (1994) 175.
[10] B. Hirrlinger, A. Stolz, H.-J. Knackmuss, J. Bacteriol. 178 (1996) 3501.
[11] N. Layh, H.-J. Knackmuss, A. Stolz, Biotechnol. Lett. 17 (1995) 187.
[12] B. Hirrlinger, 1996, A. Stolz, H.-J. Knackmuss, Deutsche Patentanmeldung, Amtszeichen 196 34 446.8/Anmeldetag 26.8.1996.
[13] B. Hirrlinger, A. Stolz, Appl. Environ. Microbiol. 63 (1997) 3390.
[14] R. Bauer, B. Hirrlinger, N. Layh, A. Stolz, H.-J. Knackmuss, Appl. Microbiol. Biotechnol. 42 (1994) 1.
[15] M.A. Cohen, J.S. Parratt, N.J. Turner, Tetrahedron Asymmetry 3 (1992) 1543.
[16] R.D. Fallon, B. Stieglitz, I. Turner Jr., Appl. Microbiol. Biotechnol. 47 (1997) 156.
[17] L. Martínková, A. Stolz, H.-J. Knackmuss, Biotechnol. Lett. 18 (1996) 1073.
[18] R. Bauer, PhD thesis, Universität Stuttgart, 1997.