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Discovery of a mandelonitrile hydrolase from *Bradyrhizobium japonicum* USDA110 by rational genome mining

Dunming Zhu, Chandrani Mukherjee, Edward R. Biehl, Ling Hua *

Department of Chemistry, Southern Methodist University, Dallas, TX 75275, USA

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Abstract

A mandelonitrile hydrolase bll6402 from *Bradyrhizobium japonicum* USDA110 was predicted by rational genome mining, i.e. combining traditional genome mining with functional analysis of the genetic organization of the putative nitrilase gene within the chromosome of microorganisms. This putative gene was cloned and over-expressed in *Escherichia coli*, and the encoded protein was purified to give a nitrilase with a molecular mass of about 37 kDa. The molecular weight of the holoenzyme was about 455 kDa, suggesting that nitrilase bll6402 self-aggregated to the active form with native structure being 12 subunits of identical size. This nitrilase was most active toward mandelonitrile with V_{max} and K_m for mandelonitrile being 44.7 U/mg and 0.26 mM, respectively. The k_{cat} and overall catalytic efficiency k_{cat}/K_m were 27.0 s^{-1} and 1.04×10^5 M^{-1} s^{-1}, indicating that nitrilase bll6402 is very active for the hydrolysis of mandelonitrile to mandelic acid. Nitrilase bll6402 also effectively hydrolyzed several mandelonitrile derivatives.

Keywords: Nitrilase; Mandelonitrile; Rational genome mining; *Bradyrhizobium japonicum*

1. Introduction

With ever-increasing environmental concerns, the development of "green methods" to produce fine chemicals is highly desirable. Biocatalysis accommodates several of the twelve principles of green chemistry defined by Anastas and Warner (1998). Intensive efforts have been made to discover new enzyme catalysts in both academy and industry. Various approaches have been developed and used to successfully discover or engineer novel enzymes (Arnold, 1998; Arnold and Volkov, 1999; Zhao et al., 2002; Robertson et al., 2004). Currently used methods, including directed evolution and metagenome approach, require screening of a large number of clones. Thus, to identify the most appropriate enzyme catalyst for a target transformation is still time-consuming, presenting a critical bottleneck in the enzyme catalyst discovery. Herein we have used a rational genome mining approach to discover an efficient enzyme catalyst for a target transformation.

Aromatic α-hydroxy carboxylic acids are valuable intermediates in pharmaceutical and fine chemical industry. For example, *p*-hydroxymandelic acid is used as a chemical adaptor system to link a tumor-targeting device with a prodrug and an enzymatic trigger for selective chemotherapy (Gopin et al., 2003; Lee et al., 2004). A straightforward method to produce them involves the hydrolysis of the corresponding α-hydroxy nitriles, which are readily accessible from addition of HCN to the corresponding aldehydes (Scheme 1) (Groger, 2001). Chemical hydrolysis of nitriles to carboxylic acids typically requires drastic conditions (strong bases or acids and relatively high reaction temperature). In this regard, biotransformation of nitriles to carboxylic acids is of particular interest (DeSantis et al., 2002, 2003; Chauhan et al., 2003; Hann et al., 2003; Martinkova and Mylerova, 2003; Hann et al., 2004). Therefore, we have been interested in seeking for highly active mandelonitrile hydrolase, which efficiently hydrolyzes aromatic α-hydroxy nitriles to α-hydroxy carboxylic acids.

Advances in molecular biology have enabled the complete sequencing of hundreds of microorganisms. The rapidly growing microbial genome sequence data obtained in the course of genome projects offer a tremendous opportunity for discovery of new enzyme catalysts (Kelly and Shockley, 2004).

* Corresponding author. Tel.: +1 214 768 1609; fax: +1 214 768 4089.
E-mail address: lhua@smu.edu (L. Hua).

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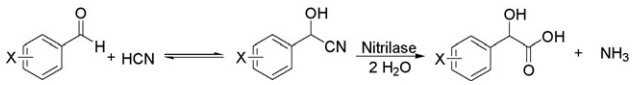

Scheme 1.

Therefore, rational genome mining, i.e. combining traditional genome mining with functional analysis of the genetic organization of the putative nitrilase gene within the chromosome of microorganisms, was used to search for putative nitrilases. A putative nitrilase gene (bll6402) from *Bradyrhizobium japonicum* USDA110 was identified to encode a protein, which might catalyze the hydrolysis of aromatic α-hydroxy nitriles to α-hydroxy carboxylic acids. This putative nitrilase gene was then cloned and expressed in *Escherichia coli*. The encoded protein was purified and characterized as an active mandelonitrile hydrolase, which showed highest activity towards the hydrolysis of mandelonitrile.

2. Materials and methods

2.1. Bacterial strains and chemicals

The *B. japonicum* USDA110 strain was obtained from Dr. Patrick Elia at USDA Soybean Genomics and Improvement Laboratory. Rosetta(DE3)pLysS from Novagen was used for expression. The nitrile substrates and the carboxylic acid standards were purchased from Sigma–Aldrich (Milwaukee, USA).

2.2. Identification of the putative nitrilase gene (bll6402) by rational genome mining

An *in silico* screening of DNA sequence database for the putative nitrilase genes in microorganisms was performed. A query using "nitrilase" as an identifier was submitted to Entrez Gene database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene) (Maglott et al., 2005) and 98 hits were obtained (as of March, 2005). Since bacteria were known to be responsible for the degradation of many nitrile compounds, the search was further limited to bacteria and the hits were eliminated to 51. Among them, 49 ORFs (open reading frames) encoded proteins containing a carbon-nitrogen hydrolase domain, which might belong to the nitrilase superfamily (Pace and Brenner, 2001; O'Reilly and Turner, 2003). Almost all the known nitrilases fell into the range of 300–385 amino acids in length (Yamamoto et al., 1992), the open reading frames encoding protein outside of this range were thus further eliminated. Sixteen ORFs encoding putative nitrilases were identified in 14 sequenced microbial genomes, as presented in Table 1. However, it was not clear which putative nitrilase possessed the desired activity, i.e. hydrolyzing aromatic α-hydroxy nitriles to carboxylic acids. It had been known that bacterial genes often are clustered based on linked functions. The genetic organization of the putative nitrilase genes within the chromosomes of microbes might shed some light on the natural function of the encoded enzymes (Podar et al., 2005). Therefore, the surrounding open reading frames of these 16 putative nitrilase genes were further examined and twelve of them were found to be flanked by either hypothetical proteins or regulator/transporter proteins. Among the other four ORFs which were flanked by putative functional genes, a putative nitrilase gene (bll6402) from *B. japonicum* USDA110 was found next to a putative mandelate dehydrogenase (bll6401) which was 55.2% identical to (*S*)-mandelate dehydrogenase from *P. putida* (Tsou et al., 1990). They were surrounded by two other putative genes encoding benzoylformate decarboxylase (blr6416) (Hasson et al., 1998) and benzaldehyde dehydrogenase (blr6417) (Denome et al., 1993) in the aromatic catabolic pathway (Latha and Mahadevan, 1997; Kaneko et al., 2002; Vela et al., 2002), and a putative gene encoding β-glucosidase (bll6177) (Grabnitz et al., 1991). This suggested that a mandelonitrile metabolic pathway as shown in Scheme 2 might exist in *B. japonicum* and mandelonitrile could be the native substrate of the putative nitrilase encoded by bll6402 gene, which might be an effective catalyst

Table 1
Organisms and locus tags of putative nitrilase genes

Organism	Putative nitrilase	Locus tag
Bordetella bronchiseptica RB50	NP_887662[a], 310 aa	BB1116
Bradyrhizobium japonicum USDA110	NP_770037, 321 aa	blr3397
	NP_773042, 334 aa	bll6402
Klebsiella pneumoniae	NP_943299, 334 aa	LV044
Neisseria gonorrhoeae FA1090	YP_208564, 304 aa	NGO1514
Nocardia farcinica IFM 10152	YP_119480, 336 aa	nfa32690
Photorhabdus luminescens subsp. Laumondii TTO1	NP_928542, 335 aa	plu1231
Pseudomonas syringae pv. tomato str. DC3000	NP_790047, 347 aa	PSPTO0189
Ralstonia solanacearum GMI1000	NP_519944, 343 aa	RSc1823
Rhodopseudomonas palustris CGA009	NP_946909, 349 aa	RPA1563
	NP_949502, 317 aa	RPA4166
Silicibacterpomeroyi DSS-3	YP_164946, 344 aa	SPOA0114
Synechococcus elongates PCC6301	YP_171411, 334 aa	syc0701_d
Synechocystis sp. PCC 6803	NP_442646, 346 aa	sll0784
Synechococcus sp. WH8102	NP_897518, 338 aa	SYNW1425
Zymomonas mobilis subsp. Mobilis ZM4	YP_162942, 329 aa	ZMO1207

[a] Accession number of putative nitrilases.