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Enzymatic nitrile hydrolysis catalyzed by nitrilase ZmNIT2 from maize. An unprecedented β-hydroxy functionality enhanced amide formation

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Abstract—To explore the synthetic potential of nitrilase ZmNIT2 from maize, the substrate specificity of this nitrilase was studied with a diverse collection of nitriles. The nitrilase ZmNIT2 showed high activity for all the tested nitriles except benzonitrile, producing both acids and amides. For the hydrolysis of aliphatic, aromatic nitriles, phenylacetonitrile derivatives and dinitriles, carboxylic acids were the major products. Unexpectedly, amides were found to be the major products in nitrilase ZmNIT2-catalyzed hydrolysis of β-hydroxy nitriles. The hydrogen bonding between the hydroxyl group and nitrogen in the enzyme–substrate complex intermediates that disfavors the loss of ammonia and formation of acyl–enzyme intermediate, which was further hydrolyzed to acid, was proposed to be responsible for the unprecedented β-hydroxy functionality assisted high yield of amide formation.

1. Introduction

Biocatalysts have attracted much attention in environmental science, synthetic organic chemistry, microbiology, and biotechnology. Hydrolytic biocatalysts are becoming optimal tools for the synthesis of pharmaceutically and biologically important molecules.[1] In particular, among hydrolytic biocatalysts, the nitrilase superfamily enzymes have evoked substantial commercial interest because of their very high synthetic potential.[2–4] Two important enzymes of this superfamily are nitrilase (EC 3.5.5.1) and nitrile hydratase (EC 4.2.1.84). Nitrilases catalyze hydrolysis of nitriles to their corresponding carboxylic acids and ammonia, while nitrile hydratases hydrolyze nitriles to amides.[5] These enzymes are highly demanding biocatalysts in drug synthesis, biodegradation of nitrile wastes, and agricultural development.[6,7] The chemical industry has considerable interest in the utilization of nitrilases for the chemo-, regio-, or enantioselective production of carboxylic acids from nitriles under mild conditions,[8–13] because chemical hydrolysis of nitriles usually requires strong basic or acidic conditions and elevated reaction temperature, that often results in the undesirable side reactions. For example, elimination of the OH group occurs in the chemical hydrolysis of β-hydroxy nitriles to yield unsaturated by-products.[14] However, the total synthetic applicability of these biocatalysts has not been fully achieved because of the paucity of available enzymes.[7,15]

Several nitrilases have been characterized from plants and microorganisms.[16–22] Recently, Glawischnig et al. cloned and characterized a nitrilase ZmNIT2 from maize (*Zea mays*), and demonstrated that it was involved in the biosynthesis of a plant hormone auxin.[23] This nitrilase was reported to convert indole-3-acetonitrile to indole-3-acetic acid at least 7–20 times more efficiently than AtNIT1/2/3 from *Arabidopsis thaliana*. The nitrilase ZmNIT2 also showed no substrate inhibition for indole-3-acetonitrile that was observed for nitrilases from *A. thaliana*.[19] This characteristic property is particularly important for enzymes to be used as an efficient catalyst in organic synthesis, because useful processes usually require high concentration of reactants to achieve optimal space productivity. These unique features stimulated us to explore the synthetic application potential of the nitrilase ZmNIT2 from maize. Therefore, we studied the substrate specificity of this nitrilase with a collection of nitriles with structural diversity. The nitrilase ZmNIT2 was found to be active toward aliphatic nitriles, phenylacetonitrile derivatives, and aromatic nitriles. In these cases, acids were obtained as the major products with concomitant formation of amides. On the contrary, nitrilase ZmNIT2 catalyzed the hydrolysis of β-hydroxy nitriles, producing amides as the major products instead of acids.

2. Results and discussion

Nitrilase ZmNIT2 gene from maize was over-expressed in *Escherichia coli* and the encoded protein was purified

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from cell-free extract by following the literature method.[23] This His-tagged enzyme was stable during storage under different conditions. For example, the lyophilized enzyme was stable for months at 4 °C. Ninety percent of the initial activity was recovered after being stored in potassium phosphate buffer (pH 7.2, 10 mM) with 50% glycerol at −20 °C for a month.

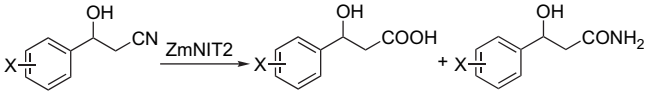
The substrate specificity of this nitrilase was studied by treating nitrile substrates with the purified enzyme in potassium phosphate buffer as described in Section 4. The products were analyzed and characterized by GC, HPLC, and NMR spectroscopy. The results are summarized in Tables 1 and 2. It can be seen that nitrilase ZmNIT2-catalyzed hydrolysis of various nitriles with diverse structures to afford the corresponding acids with concomitant amide formation. It was surprising that nitrilase ZmNIT2 efficiently catalyzed the hydrolysis of substituted benzonitrile derivatives and crotonitrile to give acids as the major products, although it showed low activity for benzonitrile (entries 1–4 in Table 1). Benzyl cyanide and its substituted counterparts were also efficiently hydrolyzed to acids with concomitant formation of amides. The amount of amide formed in the reaction was dependent on the substituent on the benzene ring. Racemic mandelonitrile was hydrolyzed by nitrilase ZmNIT2 to enantiopure (*R*)-mandelic acid.

Table 1. Hydrolysis of various nitriles catalyzed by nitrilase ZmNIT2

Entry	Nitrile	Conversion (%)[a]	
		Acid	Amide
1	Benzonitrile	Overall yield <5	
2	4-Hydroxy-3-methoxybenzonitrile	83	17
3	4-Acetylbenzonitrile	68	31
4	Crotonitrile	79	20
5	Benzyl cyanide	90	10
6	3,4-(Methylenedioxy)-phenylacetonitrile	74	26
7	2,4-Dimethoxyphenylacetonitrile	67	33
8	Mandelonitrile	45[b]	<2
9	Allyl cyanide	80	20
10	*n*-Butyronitrile	80	19
11	Valeronitrile	73	26
12	Hexanenitrile	64	36
13	Heptanenitrile	60	40
14	3-Phenylpropionitrile	84	16
15	4-Phenylbutyronitrile	83	17
16	2-Methylthioacetonitrile	76	24
17	1,4-Dicyanobutane	89	10
18	2-Methylglutaronitrile	83	17

[a] The conversion was determined by GC, HPLC, and NMR analysis.
[b] The product was (*R*)-mandelic acid with 100% ee.

Table 2. Hydrolysis of β-hydroxy nitriles catalyzed by nitrilase ZmNIT2



Entry	Substrate	Conversion (%)[a]	
		Acid	Amide
1	4-CH_3	14	85
2	4-F	17	73
3	4-Cl	13	85
4	4-Br	12	88
5	4-OCH_3	37	63
6	4-CH_3CO	27	68

[a] The conversion was determined by HPLC and NMR analysis.

As shown in Table 1, nitrilase ZmNIT2 also efficiently catalyzed the hydrolysis of aliphatic nitriles to the corresponding carboxylic acids as major products. The acid and amide product distribution was affected by the structure of aliphatic nitriles. It appeared that the amount of amide increased as the chain length became longer. Dinitriles were converted to di-acids and di-amides, and no mixed amide–acid was detected. Selective hydrolysis to mono-acid or amide was not achieved. Methylthioacetonitrile was hydrolyzed to methylthioacetic acid and methylthioacetamide.

Nitrilase-catalyzed hydrolysis of β-hydroxy nitriles results in β-hydroxy carboxylic acids, which are important precursors of β-blockers and β-amino alcohols.[14] Although biocatalytic transformation of β-hydroxy nitriles to the corresponding β-hydroxy carboxylic acids and/or amides has been achieved with nitrile hydratase/amidase-based microbial biocatalysts,[24–27] to our best knowledge, the hydrolysis of β-hydroxy nitriles catalyzed by isolated nitrilases has not been reported. Therefore, the activity of nitrilase ZmNIT2 toward a series of β-hydroxy nitriles was examined. In contrast to the nitriles in Table 1, when β-hydroxy nitriles were treated with nitrilase ZmNIT2 under the same condition the major products were amides with the conversion of 63 to 88%, along with acids as minor products (Table 2). Although the hydrolysis of mandelonitrile was highly enantioselective, nitrilase ZmNIT2 showed little enantioselectivity for the hydrolysis of β-hydroxy nitriles (less than 40% ee, data were not shown and the absolute configurations were not determined). This is consistent with the observation in most cases that a chiral carbon atom at the β-position to the reaction center would be recognized with much more difficulty than the one at α-position.[25]

There are several reports on the formation of amides as minor products in nitrilase-catalyzed hydrolysis of nitrile substrates.[15,16,28–31] The unexpected large amount of amides from β-hydroxy nitriles catalyzed by nitrilase ZmNIT2 was worthy to be further investigated. 3-(4-Fluorophenyl)-3-hydroxypropionitrile and 3-(4-methylphenyl)-3-hydroxypropionitrile were used for detailed study. To gain insight on the mechanism of amide formation, reaction mixtures were analyzed at different time intervals and the conversions of acid and amide were determined. The ratios of amide/acid in the hydrolysis of 3-(4-fluorophenyl)-3-hydroxypropionitrile after 2 and 6 h were 4.7 and 4.5, respectively, while those for hydrolysis of 3-(4-methylphenyl)-3-hydroxypropionitrile were 7.1 and 6.5. The results indicated that the ratio of product amide to acid kept fairly constant as the reaction proceeded. The same time independence of amide/acid ratio was observed for the hydrolysis of 3-phenylpropionitrile. The product mixture, which was obtained from the hydrolysis of 3-(4-fluorophenyl)-3-hydroxypropionitrile after 6 h, was incubated with a fresh enzyme overnight. The amide/acid ratio did not show meaningful change. This ruled out the possibility that enzyme deactivation was responsible for the nearly constant amide/acid ratio. The concurrent formation of amide and acid clearly suggested that the acid was not produced from amide as in the

sequential hydrolysis of nitrile catalyzed by nitrile hydratase and amidase. This was further supported by the observation that hexanoamide was not a substrate of nitrilase ZmNIT2.

We also investigated effect of temperature, pH, and solvent on the product distribution of amide and acid. The results are presented in Figures 1–3. From the results it can be seen that temperature, pH, and solvent did not exert significant impact on the product distribution of amide and acid. This is consistent with the reported independence of the amide to acid ratio on reaction conditions.[28]

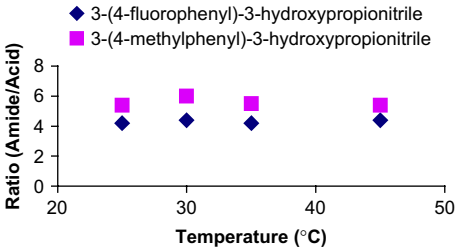


Figure 1. Temperature effect on the product distribution in hydrolysis of β-hydroxy nitriles.

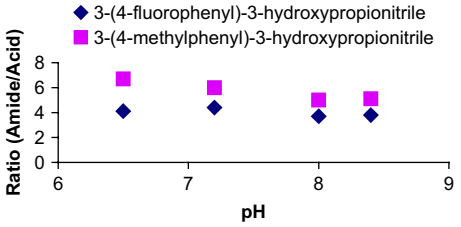


Figure 2. pH effect on the product distribution in hydrolysis of β-hydroxy nitriles.

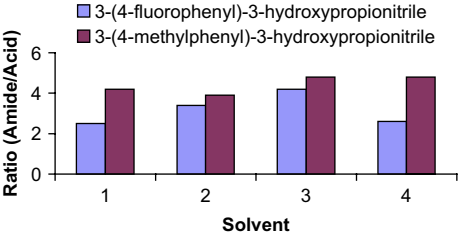
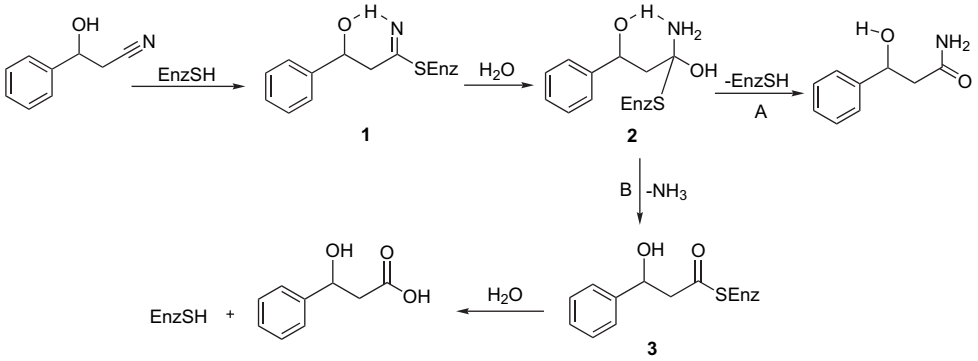


Figure 3. Solvent effect on the product distribution in hydrolysis of β-hydroxy nitriles (1. *t*-BuOH; 2. MeOH; 3. acetonitrile; 4. acetone).

Nitrilase possesses a cysteine residue in its catalytic site and the proposed reaction mechanism for the hydrolysis of nitriles involves the formation of an enzyme–substrate complex where the thiol residue of the enzyme forms a covalent bond with nitrile carbon.[29,32,33] Addition of a H_2O molecule to the complex generates a tetrahedral intermediate. Loss of ammonia from this tetrahedral intermediate gives an acyl–enzyme intermediate, which is further hydrolyzed to produce carboxylic acid and release the enzyme for next catalytic cycle. In a few cases, small amount of amide was also obtained, and it was due to cleavage of the C–S bond in the tetrahedral intermediate, that furnished amide and released enzyme.[1]

Recently, Effenberger et al. have found that amides were major products in the hydrolysis of α-fluoroarylacetonitriles and π-conjugated nitriles with acceptor groups (CN, NO_2) catalyzed by AtNIT1 from *A. thaliana*. The stabilization of the tetrahedral intermediate in the enzyme–substrate complex by these electron-withdrawing groups was proposed to be responsible for the preference of amide formation.[15] Hydrolysis of nitriles catalyzed by nitrilase ZmNIT2 should also involve participation of cysteine thiolate as a nucleophilic entity to form an enzyme–substrate complex.[29,32,33] Hydrogen bond formation is a very common feature in enzyme catalysis, when polar amine group either from substrate or from enzyme residue is in close proximity to hydroxy group from similar environment. It has also been observed that hydrogen bonding could greatly enhance the reaction rate in enzyme catalysis.[34,35] Therefore, when β-hydroxy nitriles were substrates, it was possible that the hydrogen bond between the lone pair of electrons on the nitrogen atom and the β-OH group was formed in the enzyme–substrate complex (**1**) because they were in close proximity, as shown in Scheme 1. Addition of H_2O generated the tetrahedral intermediate (**2**), which had two fates. One was loss of ammonia to form intermediate (**3**), which was further hydrolyzed to carboxylic acid (pathway B). The other one was cleavage of the C–S bond to yield amide (pathway A). These two pathways were competitive and the existence of hydrogen bond in intermediate (**2**) made pathway A favorable over pathway B, because loss of ammonia required extra energy to break down the six-membered cyclic hydrogen bond system. Therefore, amides were the major products in the hydrolysis of β-hydroxy nitriles catalyzed by nitrilase ZmNIT2. To assess the importance of intra-molecular bonding in promoting amide formation, the hydrolysis of



Scheme 1. Proposed hydrogen bonding facilitated amide formation in ZmNIT2-catalyzed hydrolysis of β-hydroxy nitriles.

4-phenylbutyronitrile and 4-hydroxy-4-phenylbutyronitrile was tested. As predicted, the carboxylic acids were the major products with amide/acid ratios being 0.2 for both substrates. Because formation of seven-membered ring was disfavored in this case, γ-hydroxyl groups did not promote the amide formation. This indicated that β-hydroxyl group played a critical role in the formation of large amount of β-hydroxy amides. Since the tetrahedral intermediate (**2**) was stabilized by intra-molecular hydrogen bonding, which was minimally affected by media, the reaction conditions did not have great effect on the product distribution as shown in Figures 1–3.

One more concern was that the tetrahedral intermediate (**2**) in Scheme 1 might be bound in a metal coordinative structure replacing the proton by a metal ion. This structure could be destructed by a strong chelating ligand such as EDTA. Thus addition of EDTA to the reaction mixture should reduce the amide formation. However, the amount of amide was not reduced when the reaction was carried out in the presence of EDTA, indicating metal ion was not involved in ZmNIT2-catalyzed nitrile hydrolysis.

3. Conclusions

Nitrilase ZmNIT2 from maize efficiently catalyzed the hydrolysis of a broad range of nitriles with diverse structures to afford the corresponding carboxylic acids and amides. For most nitriles such as aliphatic, aromatic nitriles, and phenylacetonitrile derivatives, carboxylic acids were produced as the major products. In contrast, nitrilase ZmNIT2 catalyzed the hydrolysis of *β*-hydroxy nitriles to afford amides as the major products. The unprecedented β-hydroxy functionality enhanced amide formation might be due to the hydrogen bonding between the hydroxyl group and nitrogen in the reaction intermediates, that prevented the loss of ammonia and formation of acyl–enzyme intermediate, thus led to the formation of amide as major product. Because the crystal structure of nitrilase ZmNIT2 is not known, how the intramolecular hydrogen bonding in the tetrahedral intermediate (**2**) facilitates the elimination of cysteine to give amide needs to wait for further studies.

4. Experimental

The GC analysis was performed on a Hewlett-Packard 5890 series II plus gas chromatograph. The HPLC analysis was performed on an Agilent 1100 series high-performance liquid chromatography system. ^{1}H and ^{13}C NMR spectra were recorded on a 400 MHz Bruker AVANCE DRX-400 Multinuclear NMR spectrometer. All the nitriles, amide, and acid standards were purchased from Aldrich or prepared by following the literature procedures.[14,25]

4.1. Expression of nitrilase ZmNIT2 gene in *E. coli*

Plasmid DNA containing nitrilase ZmNIT2 gene (gift from Professor Erich Glawischnig at Technische Universität München, also available from our laboratory)[23] was transformed into BL21(DE3)pLysS *E. coli* strain (Novagen). Overnight pre-cultures were diluted into LB containing 100 μg/ml of ampicillin and 34 μg/ml of chloramphenicol, the cells were induced with 0.1 mM of IPTG when optical density at 595 nm was 0.6. The bacterial cultures were incubated at 30 °C on an orbital shaker at 180 rpm for another 4 h. The cells were harvested.

4.2. Preparation of cell-free extract and purification of nitrilase ZmNIT2 enzyme

The cultures of *E. coli* BL21(DE3)pLysS were harvested by centrifugation. The cell pellet was resuspended in potassium phosphate lysis buffer (10 mM, pH 7.2, 1 mM DTT), and the cell was lysed by homogenizer. The cell-free extract was mixed with equal volume of PEI solution (0.25% polyethyleneimine MW 40–60K, 6% NaCl, 100 mM Borax, pH 7.4) to remove lipids.[36] After centrifugation, the supernatant was precipitated with 30% ammonium sulfate. The resulting precipitate was collected after centrifugation and dissolved in potassium phosphate buffer (10 mM, pH 7.2, 1 mM DTT). The lysate was desalted by gel filtration into potassium phosphate buffer (10 mM, pH 7.2, 1 mM DTT), and lyophilized.

4.3. Hydrolysis of nitriles catalyzed by nitrilase ZmNIT2

A typical experimental procedure was as follows: the nitrile (45 mM) in 1 ml of potassium phosphate buffer (100 mM, pH 7.15) was treated with 1 mg of lyophilized enzyme ZmNIT2. The reaction mixture was shaken for 14 h at 30 °C, and then acidified with a few drops of 1 N HCl solution to adjust pH to 3–4. The products and/or unreacted nitriles were extracted into 1 ml of ethyl acetate. The extract was dried over sodium sulfate and subjected to HPLC or GC analysis to determine the conversion. For GC analysis, the acids were converted to methyl ester derivatives using freshly prepared ethereal solution of diazomethane at 0 °C. For NMR analysis, the solvent was removed from the extract and the residue was dissolved in chloroform-*d* or acetone-d_6. The products were identified by comparing the retention time with authentic sample, or ^{1}H and ^{13}C NMR data with those in the literature.[14,25,37]

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