Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Molecular Catalysis B: Enzymatic 39 (2006) 156–159
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

A metal ion-based method for the screening of nitrilases

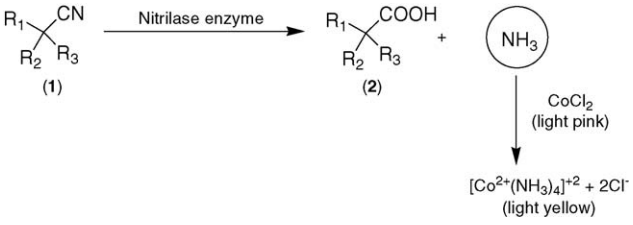
Daniel R. Yazbeck, Paulo J. Durao, Zhiyi Xie, Junhua Tao *

Pfizer, Inc., PGRD-La Jolla Laboratories, 10578 Science Center Drive, San Diego, CA 92121, United States

Available online 13 March 2006

Abstract

In this paper we describe a colorimetric method for the screening of nitrilases. When a buffered solution of $CoCl_2$ is added to a nitrilase-catalyzed hydrolysis reaction, the ammonia product forms a complex with the cobalt ion resulting in a color change from light pink to yellow, which can readily be quantified using a spectrophotometer at 375 nm. This method has been demonstrated for both wild-type and evolved nitrilases.



Keywords: High-throughput screening; Nitrilase; UV–vis spectroscopy; Enantioselectivity

1. Introduction

The use of nitrilases to produce carboxylic acids has become an increasingly attractive approach for the synthesis of pharmaceutical intermediates. This class of enzymes offers a regioselective and/or enantioselective method for nitrile hydrolyses at ambient pressure and temperature, which are important characteristics for their development as industrial catalysts [1,2].

In order to develop efficient processes using nitrilases, it is many times important to screen libraries of engineered proteins that will reveal new enzyme variants exhibiting the desired reactivity as well as selectivity. This can be very time consuming if conventional analytical methods of screening such as HPLC, LC–MS, CE and GC are employed. Hence, there is a need to develop rapid screening methods in order to identify the ideal nitrilase candidates that can be used to carry out a desired process.

There are several methods currently available for nitrilase screening [3–6] and among them one of the most popular is a fluorescent assay based on the reaction of ammonia with a buffered *o*-phthaldialdehyde-2-mercaptoethanol solution [3]. Although the method is quite sensitive ($\varepsilon = 1.7875 \times 10^5\ M^{-1}\ cm^{-1}$), the buffer is not stable and the process requires a 30 min development time, which can be time-consuming in screening libraries with millions of variants.

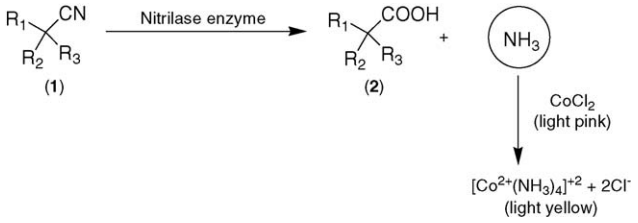
In this paper we describe a simple spectroscopic method for the rapid screening of nitrilases. As shown in Scheme 1, during a nitrilase reaction, one molecule of a nitrile (**1**) is converted to one molecule of acid product (**2**) and one molecule of ammonia. When a buffered solution of $CoCl_2$ is added to the reaction mixture, the resulting ammonia forms a complex with the cobalt ion resulting in a color change from light pink to yellow. This color change can easily be quantified using a spectrophotometer at 375 nm.

Although in principle a variety of metal ions could be used (including cobalt, nickel and copper to name a few), cobalt chloride was ideal due to a variety of reasons that will be described. This method has been validated for the screening of several nitrile substrates as well an evolved library of nitrilases.

The use of this method offers a few advantages over the nitrilase assays that have been described in the literature. First, it has a shorter development time. The addition of aqueous ammonia to a solution of a cobalt chloride will convert the hydrated cobalt ion into an ammonia complex in no more time than it takes to

* Corresponding author. Tel.: +1 8585184484; fax: +1 8586788199.
E-mail address: alex.tao@BioVerdant.com (J. Tao).

1381-1177/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2006.01.036



Scheme 1. The nitrilase-catalyzed reaction and spectrophotometric assay.

mix the solutions together. Second, the issue of buffer stability, which is a prominent factor in the fluorescent assay described above, is not a concern with this method.

2. Materials and methods

2.1. Materials

All chemicals of the highest purity were purchased from Aldrich (Milwaukee, WI), Fluka (Buschs, Switzerland) and Sigma (St. Louis, MO). Nitrilase Nit102 was obtained from Biocatalytics (Pesadena, CA). The *Arabidopsis thaliana* nitrilase 1 (AtNit1) gene was cloned from a cDNA library. BugBuster and Benzonase Nuclease were purchased from Novagen (Madison, WI). A 10 mM indicator solutions ($NiCl_2$, $CuCl_2$ and $CoCl_2$) were prepared by dissolving the appropriate weight of metal reagent in 10 mM of Tris buffer at pH 7.0. BES buffer (BES dissolved in water), nitrilase buffer (potassium phosphate buffer, 1 mM EDTA, 2 mM DTT) and Tris buffer (Tris dissolved in water) were all prepared at 10 mM and pH 7.

2.2. Protein expression and preparation

E. Coli cells harboring a plasmid expressing the *A. thaliana* nitrilase 1 (AtNit1) gene were grown in LB medium with ampicilin (100 μg/ml) at 37 °C overnight, which was used (5–20%) to inoculate an induction culture. Protein expression was induced after 3 h incubation at 37 °C by adding IPTG to a final concentration of 0.2 mM. The cells were then cultured at 30 °C for 16–18 h, harvested by centrifugation and then lysed by adding BugBuster with Benzonase. The nitrilase reaction was set in a 1 mL block with 150 μL of 10 mM Tris buffer (pH 7), 20 μL of cell lysate and 10 μL of a substrate stock solution (100 mg/mL in DMF) and left shaking for 2 h at 650 rpm at room temperature. After 2 h, 50 μL of the mixture was taken to a UV–vis plate and 10 μL of the sample to a fluorescence plate. For the UV–vis assay, 50 μL of $CoCl_2$ was added to the sample and for the fluorescence method, 150 μL of the fluorescence buffer was added. The resulting solution was mixed by shaking at 700 rpm.

2.3. Determination of nitrilase activity

Four milligrams of nitrilase Nit102 was added to a substrate solution (20 mg in 1.0 mL of 10 mM Tris buffer at pH 7.0). After 2 h, 50 μL of the reaction solution was sampled in a 96 well plate (BD4343), and quenched with 50 μL of $CoCl_2$/Tris solution (10 mM Co in 10 mM Tris buffer, pH 7). The resulting mixture was allowed to develop for 5 min and then analyzed by a spectrophotometer at 375 nm.

3. Results and discussion

3.1. Selection of metal ion and buffer system

Three metal cations were tested ($CuCl_2$, $CoCl_2$ and $NiCl_2$) in three different buffers (BES, Tris, KPB). The use of buffers is customary at neutral pH to carry out reactions and compatible with the activity profile of the majority of nitrilases. It was not possible to work with a non-buffered metal ion/water system since the color indicator is pH dependent. Furthermore, a green precipitate was observed at a pH above 8. The use of potassium phosphate buffer (10–100 mM) was not pursued because it forms precipitates with all three metal ions. In order to determine the optimum metal ion/buffer system, we first set out to determine which system has the highest sensitivity in detecting ammonia by evaluating the difference of absorbance between the metal ion/ammonia buffered complex and the metal ion indicator control (Fig. 1).

The difference in absorbance between the $CoCl_2$/ammonia complex solution and the $CoCl_2$ solution in 10 mM Tris buffer was clearly the highest (Fig. 1). The results also show that $NiCl_2$ with either BES or Tris buffer exhibits a difference in absorbance of about 0.2 units, which is not sufficient to be used as reliable colorimetric method. Although the $CuCl_2$ system showed a maximum difference in absorbance of 2.4 and 1.5 in Tris and BES, respectively, it was later determined to have a very high background made it less ideal than the $CoCl_2$ indicator (data not shown). Nevertheless, both $CoCl_2$ in BES and $CuCl_2$ in either Tris or BES buffers could be used as colorimetric indicators for the detection of ammonia.

3.2. Wavelength of detection

In order to choose the ideal detection wavelength (λ), UV–vis spectra of the $CoCl_2$ indicator and $CoCl_2/NH_3$ complex were captured from 190 to 990 nm (Fig. 2A). A difference

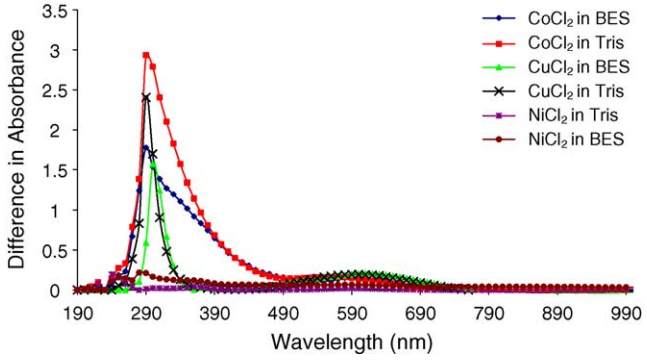


Fig. 1. Evaluation of the metal/buffer systems for their capacity to detect ammonia. The graph illustrates the differences in absorbance between the absorbance obtained for the metal/ammonia complex solution and the metal solution control (without ammonia). A 10 mM $NiCl_2$, $CoCl_2$ and $CuCl_2$ in 10 mM of BES or Tris were tested at pH 7.

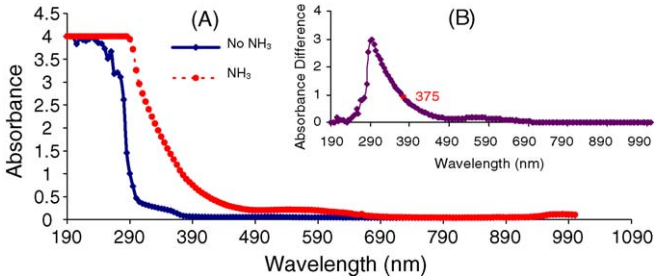


Fig. 2. (A) Absorbance spectra of the 10 mM $CoCl_2$ in 10 mM Tris at pH 7 with and without ammonia. (B) Difference in absorbance obtained by subtracting the values of the two spectrums shown in (A).

in absorbance between the two spectra can be seen in Fig. 2B, and from both graphs it was possible to determine the ideal wavelength.

As can be seen from Fig. 2A, the largest difference in absorbance occurs at 290 nm. However, any wavelength between 190 and 375 nm will result in a strong background (absorbance greater than zero) for the non-complexed $CoCl_2$ (blue line, Fig. 2A). Any wavelength between 375 and 490 nm will be suitable for the detection of ammonia due to a low background (close to zero). A wavelength of 375 nm was therefore chosen to achieve the highest difference in absorbance (0.972 absorbance units) while still maintaining a low background (0.084 absorbance units).

3.3. Calibration curve

A calibration curve ranging from 0 to 100 mM ammonia was determined (Fig. 3). The absorbance at 375 nm increased linearly ($R^2 = 1$) with increasing concentration of ammonia (0–100 mM) with a calculated extinction coefficient being $9.86 \times 10^3\ M^{-1}\ cm^{-1}$. The background inherent to the method (caused by the indicator) is close to 0.1 absorbance units. Therefore the method is not able to differentiate concentrations of ammonia below 5 mM. The linear functional range of the assay is thus between 5 and 100 mM.

3.4. Establishing and validating the screening method

To prove that this essay could be used for the screening of nitrilases, we chose nitrilase from *A. thaliana* and hydrocinnamonitrile as the substrate (Scheme 2). The reaction was allowed

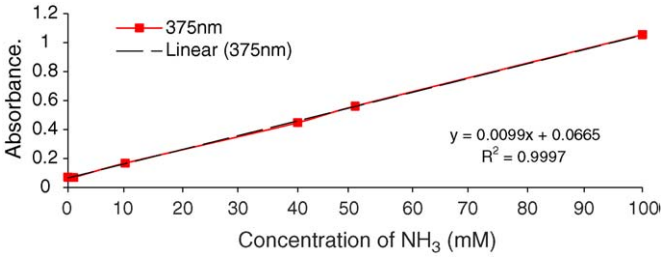
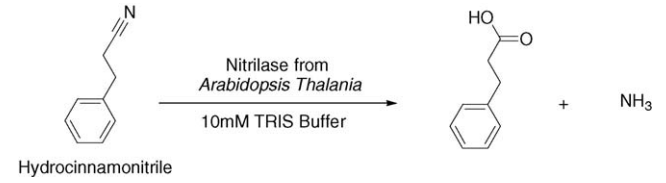


Fig. 3. Calibration curve of a 10 mM solution of $CoCl_2$ in 10 mM Tris at pH 7 in different ammonia concentrations. The absorbance was detected at 375 nm.



Scheme 2. Reaction of Nit102 and hydrocinnamonitrile (1 mg/mL of the nitrilase, 20 mg/mL of substrate in 10 mM Tris buffer at pH 7.0). The reaction was monitored by HPLC and allowed to proceed to 100% conversion.

to proceed to completion as confirmed by HPLC. Samples of different volumes were taken corresponding to 0, 1.5, 3, 6, and 12 μmol of NH_3, and quenched with the appropriate volume of 10 mM $CoCl_2$/Tris indicator solution to reach a final volume of 100 μL. As can be seen from Fig. 4, although the absorbance observed for each concentration of ammonia is slightly less than that of the theoretical absorbance based on the calibration curve (Fig. 3), the results clearly show a linear correlation of ammonia generated from the reaction.

3.5. Application to directed evolution

Two AtNit1 variants, P54C6 and P70F5, have been identified with increased activity compared to the wild-type enzyme on isobutylsuccinonitrile (IBSN) from previous screening. *E. Coli* cells expressing wild-type, two mutants- and a negative control with no nitrilase were cultured and expressed. A 20 μL of lysate from each expressed enzyme was used to carry out several reactions with two substrates, hydrocinnamonitrile and isobutylsuccinonitrile. After 2 h, 50 μL of the mixture was quenched with 50 μL of $CoCl_2$ and analyzed via UV–vis while 10 μL of the same sample was transferred to a fluorescence plate and quenched with 150 μL of fluorescence buffer (buffered *o*-phthaldialdehyde-2-mercaptoehtanol solution). The endpoint prior to measurement for the $CoCl_2$ and fluorescent samples were taken after a 2 and 30 min incubation time, respectively. The relative activity profiles of various strains obtained from either the UV or fluorescent method of detection were very

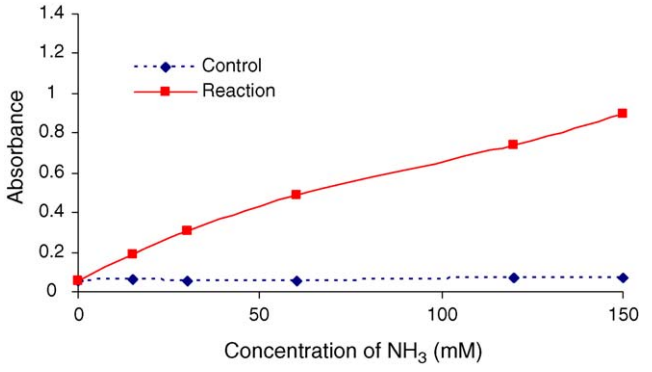


Fig. 4. The reaction contained 1 mg/mL of the nitrilase and 20 mg/mL of hydrocinnamonitrile in 10 mM Tris buffer at pH 7.0. The control reaction contained no enzymes. All three control reactions (1, indicator solution; 2, indicator solution/substrate; 3, indicator solution/enzyme) showed similar data to the control reaction with absorbance values less than 0.2.

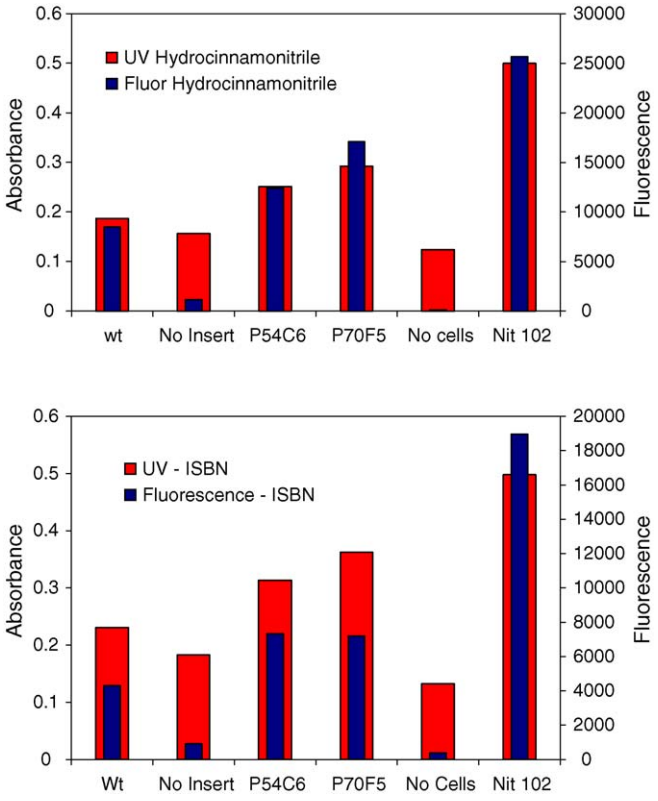


Fig. 5. Results from the UV assay (light blue) and the fluorescence assay (dark blue) for the screening of a wild type (Nit06), a mutant with no nitrilase insert (negative control), two mutants with higher activity, a positive control (Nit102) and another negative control (no cells).

comparable in being able to differentiate nitrilase activity under these conditions (Fig. 5). These charts further revealed that both evolved strains, P54C6 and P7F5, showed an increase in activity towards both substrates as compared to the wild-type enzyme. It is important to note, however, that the signal to noise ratio resulting from cells not expressing the enzyme in the fluorescent assay described by Banerjee et al. is smaller than that of the UV assay described in this paper.

In summary, a new method for nitrilase screening has been developed to detect nitrilase activity. Furthermore, preliminary data in our lab has shown that the activity of nitrilases is not affected when a nitrilase-catalyzed reaction was carried out in the cobalt chloride indicator solution. As such, this assay has the potential to be used for the continuous monitoring of the production of ammonia over time in a nitrilases-catalyzed reaction. Once fully validated, this added feature can offer a significant enhancement in the analytical toolboxes for the real-time monitoring of nitrilase-catalyzed reactions.

Acknowledgments

The authors gratefully acknowledge Shanghui Hu, Carlos Martinez, Sven Tummers and Anita Gujral for their general lab support and guidance.

References

[1] L. Martinkova, V. Mylerova, Curr. Org. Chem. 7 (2003) 1279–1295.
[2] C. O'Reilly, P.D. Turner, J. Appl. Microbiol. 95 (2003) 1161–1174.
[3] A. Banerjee, R. Sharma, U.C. Banerjee, Biotechnol. Appl. Biochem. 37 (2003) 289–293.
[4] A. Banerjee, P. Kaul, R. Sharma, U.C. Banerjee, J. Biomol. Screening 8 (2003) 559–565.
[5] M.R. Dadd, D.C. Sharp, A.J. Pettman, C.J. Knowles, J. Microbiol. Methods 41 (2000) 69–75.
[6] G. DeSantis, K. Wong, B. Farwell, K. Chatman, Z. Zhu, G. Tomlinson, H. Huang, X. Tan, L. Bibbs, P. Chen, K. Kretz, M.J. Burk, J. Am. Chem. Soc. 125 (2003) 11476–11477.