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Comparison of Cyanide Degrading Enzymes Expressed from Genes of Fungal Origin

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Abstract

A variety of fungal species are known to degrade cyanide through the action of cyanide hydratase, a specialized nitrilases which hydrolyze cyanide to formamide. This work is a report on two unknown and uncharacterized members from *Neurospora crassa* and *Aspergillus nidulans*. Recombinant forms of three cyanide hydratases (CHT) originated from *N. crassa, Gibberella zeae, and A. nidulans* were prepared after their genes were cloned with N-terminal hexahistidine purification tags, expressed in *E. coli* and purified using immobilized metal affinity chromatography. These enzymes were compared according to their pH activity profiles, and kinetic parameters. Although all three were similar, the *N. crassa* CHT has the widest pH range of activity above 50% and highest turnover rate (6.6×10^8 min^{-1}) among them. The CHT of *A. nidulans* has the highest K_m value of the three nitrilases evaluated in here. Expression of CHT in both *N. crassa* and *A. nidulans* were induced by the presence of KCN, regardless of any presence of nitrogen sources. These data can be used to determine optimal procedures for the enzyme uses in the remediation of cyanide-containing wastes.

Key Words : Cyanide hydratase, Fungal gene, Enzyme kinetics

1. Introduction

Cyanide waste is becoming an increasingly prevalent problem in today's society. An estimated 18 billion liters of cyanide containing waste are annually generated in the United States[1]. With such uses as gold and silver mining, electroplating, steel manufacturing, polymer synthesis, pharmaceutical production, and other specialized applications including dyes, and agricultural products, it is difficult to avoid its use. Remediation of cyanide containing waste is necessary due to the ability of cyanide to poison the respiratory system by inhibiting the final transport of electrons from cytochrome C oxidase to oxygen. Currently, the most widely used methods for detoxification are chemical oxidation of cyanide to less toxic compounds of stabilization using reagents and cement in soil to reduce permeability.

A variety of biological approaches to cyanide waste remediation have been proposed over many decades[2]. The use of microbial nitrilases presents the most likely biological approach. There are enzymes that convert cyanide to non-toxic products, but special conditions are required for this conversion. Nitrogenases, which are found in nitrogen-fixing prokaryotes, require strictly anaerobic conditions[3]. Rhodanese, which is an enzyme found in all animals, some plants, fungi, and prokaryotes, requires thiosulfate to function[4]. Nitrilases do not have these drawbacks. They convert cyanide using a simple hydrolytic pathway which involves nucleophilic attack by a conserved cysteine[5]. This cysteine, along with lysine and glutamic acid are conversed

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throughout the nitrilase superfamily and formed the catalytic triad necessary for catalytic activity[6,7]. It was observed that there are a number of reactions catalyzed by nitrile-metabolizing enzymes that are either already used in large scale or have the potential to become so. This has been demonstrated by the success of such organisms as *Pseudomonas chlororaphis* B23[8] and *Rhodococcus rhodochrous* J1, which have been used commercially to successfully convert another nitrile, 3-cyanopyridine, to nicotinic acid[9].

Microbial nitrilases are a family of enzymes that convert nitriles, such as cyanide, to less harmful compounds through a hydration reaction[10]. Cyanide hydratases (CHT), which are found in numerous plant pathogenic fungi such as *Fusarium solani* and *Gloeocercospora sorghi*, convert cyanide to formamide[11]. Other cyanide hydratases are found in *Fusarium lateritium*[11,12], and *Leptosphaeria maculans*[13]. The related bacterial nitrilases, cyanide dihydratases, convert cyanide to formate and ammonia[14] and are found in *Alcaligenes xylosoxidans* subsp. *Dentificans*, *Bacillus pumilus*, and *Pseudomonas stutzeri* AK61. It has been proposed that subtle differences in the active site dictate the leaving group, and hence distinguish the cyanide dihydratases from the hydratases[15]. Both groups of enzymes have many qualities that make them promising candidates for remediation. They have stability over long periods, require no co-factors, function as purified enzyme, crude extracts, or within cells, and are readily expressed at high levels.

Using genome gazing we have observed that a variety of non-plant pathogenic fungi, including human pathogens such as *A. fumigatus*, carry related genes. We have cloned the genes from strains of *A. nidulans*, *G. zeae* and *N. crassa*.

The many uses of cyanide produce a wide variety of waste products with varying qualities, such as concentration of other contaminants and pH. This work compares these three cyanide hydratases with respect to their relative stabilities, kinetic parameters, and ability to work under pH conditions expected for cyanide containing wastes.

2. Materials and Methods

2.1. Strains and plasmids

The plasmids and strains used were listed in Table 1. The *N. crassa* knockouts were provided by the Fungal Genetics Stock Center.

2.2. Culture media and reagents

E. coli strains were grown in LB broth containing 0.05% glucose, 0.5% glycerol, and 0.2% lactose (for protein expression) which is essentially the auto-induction media of Studier[16]. Antibiotics were added to concentrations of 100 μg/mL ampicillin, 25 μg/mL chloramphenicol, and 25 μg/mL kanamycin, for selection in *E. coli* strains. *N. crassa* strains were grown in Vogel's medium[17]. *A. nidulans* and *G. zeae* were grown in complete medium[18].

2.3. DNA manipulations

The cyanide hydratase gene was amplified from *A. nidulans* and *G. zeae* genomic DNA and cDNA form *N. crassa* using PfuTurbo Hotstart PCR Master Mix (Stratagene, La Jolla, USA). The primers used introduced an *Nde I* site at the ATG codon and a unique site in the downstream primer for cloning into p2160. These clones were sequenced and later moved by subcloning into pET26b to produce untagged protein and pET28a to generate N-terminal His-tagged proteins.

Removal of introns was essential for the genomic *A. nidulans* and *G. zeae* clones. The *A. nidulans* gene

Table 1. Plasmids and strains used in this work

Plasmid/strain	Description	Reference
E.coli B1	BL21(DE3)pLysS F⁻ *ompT hsdS$_B$ gal dcm*	Novagen
E.coli B2	MM294 lacking *laclq lacZ* ΔM15	This work
E.coli B2N	B1, pET28a *NdeI-EcoRI* of *N. crassa* CHT	This work
E.coli B2G	B1, pET28a *NdeI-HindIII* of *G. zeae* CHT	This work
E.coli B2A	B1, pET28a *NdeI-EcoRI* of *A. nidulans* CHT	This work

contained three introns and *G. zeae* contained two. The Quick Change Site Directed Mutagenesis Kit (Stratagene, La Jolla, USA) was used to create deletions of the introns, producing the predicted proteins as shown in Fig. 1 (A.A. sequences). All constructs were transformed into *E. coli* for routine cloning and expression was achieved in BL21(DE3)pLysS.

2.4. Expression and purification of CHT from *E. coli*

Protein production from the three mutant *E. coli* strains was achieved in 50 mL cultures of auto-induction media containing 25 μg/mL kanamycin, 0.5% glycerol, 0.05% glucose, and 0.2% lactose[16] and grown overnight at 30°C.

After cell harvest, cell pellets were in resuspended in 0.02M sodium phosphate buffer with 0.1 M NaCl, 0.0125 M imidazole and 1 mg/mL lysozyme. Cells were lysed by freezing-thawing to produce crude lysate without DNA.

The histidine-tagged CHT enzymes were purified from crude cell lysates by immobilized metal affinity chromatography using a 1 mL precharged HisTrap Ni Sepharose HP Column (Amerisham Bioscience, Piscataway, USA). Enzymes were eluted using 10 volumes of imidazole buffer. Peak fractions were collected and stored at 4°C.

2.5. pH activity

The pH profiles for the purified cyanide hydratases of *G. zeae, N. crassa,* and *A. nidulans* were determined using sodium phosphate buffers between pH 4.5 to 11. A 1M stock of KCN was diluted in 100 mL MOPS, pH 7.6 to yield a final KCN of 100 mM. Reactions were run in duplicate at room temperature in a final 300 μL having 100 mM buffer, enzyme and 10mM KCN. Enzyme concentrations were 1.8 μg/mL, 2.2 μg/mL, 6.9 μg/mL for *N. crassa, A. nidulans,* and *G.*

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N. crassa     --MVLTKYKAAAVTSEPCWFDLEGGVRKTIDFINEAGQAGCKLVAFPEVWIPGYPYWMWK  58
G. zeae       --MVLTKYKAAAVTSEPCWFDLEGGVRKTIDFINEAGQAGCKLVAFPEVWIPGYPYWMWK  58
A. nidulans   MSPVLKKYKAAAVNAEPGWFDLEESVRRTIHWINEAGRNRCKLIAFPELWIPGYPYWMWK  60

N. crassa     VTYQQSLPMLKKYRENAMAVDSDEFRRIRRAARDNQIYVSLGFAEIDHATLYLAQALIDP  118
G. zeae       VTYLQSLPMLKRYRENSMAVDSEEMRRIRRAARDNQIFVSLGFSEIDHATLYLSQVLIGP  118
A. nidulans   VNYQESLPLLKKYRENSLLSDSEEMRRIREAARANKIYVSLGYSEVDLASLYTTQVLISP  120

N. crassa     TGEVINHRRKIKPTHVEKLVYGDGAGDTFMSVTPTELGRLGQLNCWENMNPFLKSLNVSM  178
G. zeae       DGAVINHRRKIKPTHVEKLVYGDGAGDTFMSVSETEIGRVGQLNCWENMNPFLKSLNVSA  178
A. nidulans   AGNILNHRRKIRATHVERLVFGDGTGDTTESVVQTEIGRVGHLNCWENMNPFMKSYAASL  180

N. crassa     GEQIHIAAWPIYPGKETLKYPDPATNVADPASDLVTPAYAIETGTWTLAPFQRLSVEGLK  238
G. zeae       GEQVHVAAWPVYPGKERQVYPDPATNYADPASDLVTPEYAIETGTWTLAPFQRLSVEGLK  238
A. nidulans   GEQVHIAAWPLYPGKETLKYPDPYTNVAEANCDLVTPAYAIETGTYTLAPWQTITEEGIK  240

N. crassa     KNTPEGVEPETDPSTYNGHARIYRPDG-SLVVRPDKDFDGLLFVDIDLNECHLTKALADF  297
G. zeae       INTPEGVEPETDPSVYNGHARIYRPDG-SLVVKPEKDFDGLLFVDIDLNECHLTKVLADF  297
A. nidulans   LNTPPG-KPLEDPNIYNGHGRIFAPDGRNLVPHPAKDFQGLLYVDIDLDEIHLTKSLADF  299

N. crassa     AGHYMRPDLIRLLVDTSRKELVTEVD--RNGGIVQYSTRERLGLNTPLEND--KEGKK--  351
G. zeae       AGHYMRPDLIRLLVDTRRKELITEAD--PNGSIATYSTRQRLGLDKPLEK--KEGEDTP-  352
A. nidulans   GGHYMRPDLIRLLVDGNRKDLVVSEDR-INGGIKYTSTMDRVGLTKPLEAP---KPTDQK  355

N. crassa     ----------
G. zeae       DVL-------- 355
A. nidulans   E---------- 356
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Fig. 1. Alignment of sequences of the three cyanide degrading nitrilases. The catalytic triads residues are underlined.

zeae, respectively. Reactions were run for 60 min with the baseline sample taken at 1 min. The picric acid method for determining cyanide concentration was used to determine the rate of degradation[19]. The absorbances at 520 nm were made remained cyanide.

2.6. Enzyme kinetics

Enzyme was diluted in 1 mL 0.1 M MOPS, pH 7.4 and KCN added to final concentrations of 2, 5, 10, 20, 30, 40 and 50 mM. Reactions were run for 10 min at room temperature with samples taken at 1 and 10 min. Enzyme concentrations used for kinetic analysis were 0.18 μg/mL for *N. crassa*, 0.44 μg/mL for *A. nidulans* and 0.69 μg/mL for *G. zeae*. The assays otherwise performed as described for the pH profiles. Through a general enzymatic kinetic analysis V_{max} and K_m were obtained.

2.7. Cyanide induction in fungal cultures

A. nidulans cultures were prepared in 2 mL complete medium. After growing overnight, the culture was diluted by 5 fold to 1 mL in two separate tubes. KCN was added to 0.1 mM to one tube for induction. Ammonium nitrate (10 mM) as NH_4^+ source was also used for induction.

N. crassa cultures of the wild type and a mating type CHT knockout strain were grown overnight at 30°C in Vogel's medium containing 1.5% glucose. The 2 mM KCN was added to each tube. After growing 3 h at 30°C, the cultures were harvested and lysed with sonication. Each lysate was assayed with 5 mM KCN.

3. Results and Discussion

3.1. Cloning for expression in *E.coli* and purification of CHT

The CHT genes of *N. crassa, A. nidulans,* and *G. zeae* were PCR-amplified from a cDNA library (*N. crassa*) or the genomic DNAs, respectively. The resulting products were then subcloned into p2160 and transformed into *E. coli* to allow for blue-white screening. Introns were deleted through a site-directed mutagenesis. Then, they were subcloned using the *Nde I* site of pET28a to create N-terminal His-tagged constructs and likewise in pET26b to generate untagged proteins. The final confirmed constructs were transformed into BL21(DE3)pLysS via electroporation.

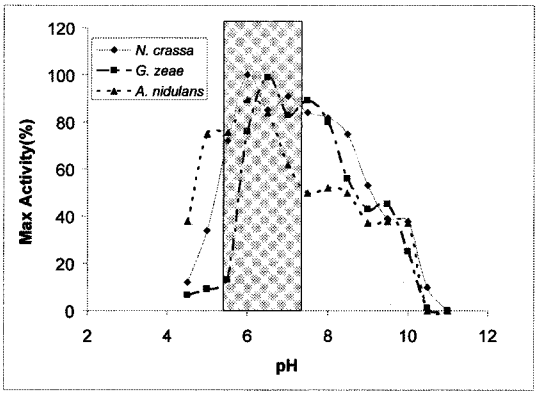

Fig. 2. CHT activity change over pH for three strains, *N. crassa, G. zeae, and A. nidulans*. The shaded bar represents their common maximum activity range.

All three sequences, in Fig. 2 were active against cyanide. The DNA of each clone was verified. Overexpression was also visualized using SDS-PAGE by comparing to a positive or negative control (not shown). Expression levels and enzyme activity were comparable from both the tagged and untagged versions.

The CHT enzymes were purified using immobilized metal affinity chromatography, and SDS-PAGE was used to analyze the purity and relative concentration of the protein preparations. The enzymes were stable for months at 4°C.

3.2. pH activity profiles

The pH activity profiles of purified CHTs were measured at pH range of 4.5 to 11 (Fig. 2). All of these displayed maximum activity between pH 6-7. The CHT from *G. zeae* displayed greater than 50% maximal activity in the range of pH 6-8.5, the narrowest range of activity. *N. crassa* CHT had greater than 50% maximal activity over the widest range, from pH 5 to 9. *A. nidulans* CHT showed the lowest activity (about 80% of the others), though the range of activity was relatively wide.

3.3. Kinetic analysis

The values for K_m, V_{max} and k_{cat} of the purified re-

Table 2. CHT enzyme kinetic parameters determined in this work

Enzyme Source	$K_m(mM)$	$V_{max}(mMmin^{-1})$	$k_{cat}(min^{-1})$	k_{cat}/K_m $(mM^{-1}min^{-1})$
N. crassa	16.2	2.7	6.6×10^8	4.07×10^7
G. zeae	20.3	0.8	2.0×10^7	9.85×10^5
A. nidulans	32.2	1.35	3.4×10^8	1.06×10^7

Table 3. Induction of CHT activity in *N. crassa* (10 mM NH_4NO_3; 2 mM KCN; * provided by Fungal Genetics Stock Center)

Strain	*Inducer: NH_4^+*	+	+	-	-
	Inducer: KCN	-	+	-	+
*FGSC2489		< 0.1	10	< 0.1	6.7
*FGSC9718		0.7	6.7	< 0.1	7.0

combinant CHT enzymes are listed in Table 2. While all three enzymes are relatively similar, the *N. crassa* CHT has the highest V_{max} and k_{cat} values, but it has a K_m two-fold lower than *A. nidulans*. It seems that the *G. zeae* has the lowest activity of the group, although the K_m is relatively high, since the turnover number is extremely low. This is also true for the CHT of *A. nidulans*, which has the highest K_m value of the three. That is, k_{cat}/K_m values stand in the following order: *N. crassa, A. nidulans and G. zeae*; *N. crassa* has the most effective enzyme system among the three, considering enzymatic activity as well as substrate affinity.

It should be noted that the difference was only 3-fold between the highest and lowest V_{max} values whilst that in the turnover rate was by more than 30-fold.

3.4. Regulation of CHT expression

Cultures of *N. crassa* wild type and a knockout mutant were grown in minimal media as described. The effect of cyanide was tested by adding 2mM KCN to each set of cultures. The effect of exogenous ammonia was also tested for *N. crassa* because the breakdown of cyanide leads to the release of ammonia. Table 3 shows the change in OD per min. It appears that while the presence or absence of nitrogen has no effect on induction of activity, the presence of cyanide during growth clearly draws induction of CHT gene expression. The CHT knockout strains had no detectable activity.

Other fungal nitrilases have previously been studied such as from *F. lateritium, F. solani, F. oxysporum* and *L. maculans*. Cyanide hydratase activity in all three of these organisms has been shown to be inducible by cyanide[11,13,20]. This work demonstrated that enzymes with similar activity and regulation are found in typical strains of *N. crassa* and *A. nidulans*. The amino acid sequence encoded by the CHT of *N. crassa* is 72% and 82% identical to the sequences of *G. sorgi* and *F. lateritium*, respectively. That of *A. nidulans* is found to be about 60% identity to the others, which is the most distant among the three.

4. Conclusions

Comparisons of the notable fungal nitrilases will help to determine the best candidate for the remediation of cyanide-containing industrial wastes. Based on our results, the CHT from *N. crassa* is the most promising one for this purpose. It reveals high activity over a wide range of pH values, a high turnover rate and V_{max}.

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