Cyanide hydrolysis in a cyanide-degrading bacterium, *Pseudomonas stutzeri* AK61, by cyanidase

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The cyanide-degrading bacterial strain AK61 was isolated from waste water at a metal-plating plant. The isolated strain was characterized by Gram-staining, quinone analysis, fatty acid profile and the API 20NE identification system, and identified as *Pseudomonas stutzeri*. Whole cells were able to degrade cyanide rapidly in a 1 mM solution containing no organic substances, and produced ammonia as a product. The induction of the cyanide-degrading activity of *P. stutzeri* AK61 did not depend on the presence of cyanide in the culture medium during growth. The cyanide-degrading enzyme was purified approximately 49-fold from a cell extract of *P. stutzeri* AK61. The enzyme had a K_m of 1.7 mM for cyanide and a specific activity of 54.6 μmol ammonia produced min^{-1} . The activity of the enzyme was optimal at 30 °C and pH 7.5. The results of SDS-PAGE, gel-filtration chromatography and NH_2 -terminal amino acid sequence analysis of the enzyme indicated that the functional enzyme was an aggregated protein consisting of a 38 kDa polypeptide. Like cyanidase (cyanide dihydratase), it was shown that the enzyme catalysed the hydrolysis of cyanide to ammonia and formate.

Keywords: cyanide degradation, hydrolysis, cyanidase

INTRODUCTION

Cyanide is highly toxic to living organisms because it inactivates the respiration system by tightly binding to cytochrome *c* oxidase (Solomonson & Spehar, 1981). Despite the toxicity of cyanide, large amounts of it are used in the metal-plating, pharmaceutical and agricultural-chemical industries. Waste water containing cyanide from these industries must be treated so that it contains a low level ($<1 \text{ mg l}^{-1}$) of cyanide before being released into the environment (Raybuck, 1992). Currently, cyanide in waste water is removed by chemical methods such as alkaline chlorination, ozonization and wet-air oxidation (Raybuck, 1992). These methods are expensive and require the use of hazardous reagents such as chlorine and sodium hypochlorite. Biological treatment of cyanide may be cheaper and more environmentally acceptable than chemical methods (Dubey & Holmes, 1995).

To develop the biological treatment of cyanide, screening of cyanide-degrading micro-organisms has been carried out. Some biological pathways have been

suggested for the degradation or detoxification of cyanide in bacteria and fungi (Knowles & Bunch, 1986; Raybuck, 1992; Dubey & Holmes, 1995). *Pseudomonas fluorescens* NCIMB 11764 has been shown to grow in a medium containing cyanide as the sole nitrogen source (Harris & Knowles, 1983). *P. fluorescens* NCIMB 11764 converts cyanide to ammonia and carbon dioxide. This reaction may proceed by means of NAD(P)H-dependent oxygenase and cyanase (Dorr & Knowles, 1989). A *Pseudomonas* species which degraded cyanide by another pathway was isolated from the activated sludge at a coke plant (White *et al.*, 1988). Although the cyanide-degrading enzyme was not purified, it was shown that the strain degraded cyanide to ammonia and formate under both aerobic and anaerobic conditions. Recently, *Alcaligenes xylosoxidans* subsp. *denitrificans* DF3 (Ingvorsen *et al.*, 1991) and *Bacillus pumilus* C1 (Meyers *et al.*, 1991) were also shown to degrade cyanide by the same pathway as the pseudomonad. These strains have been reported to degrade cyanide by cyanidase (cyanide dihydratase), which catalyses the hydrolysis of cyanide to ammonia and formate (Ingvorsen *et al.*, 1991; Meyers *et al.*, 1993). Some phytopathogenic fungi,

including *Gloeocercospora sorghi* (Fry & Munch, 1975; Wang *et al.*, 1992), *Fusarium lateritium* (Cluness *et al.*, 1993) and *Stemphylium loti* (Fry & Millar, 1972), have also been reported to degrade cyanide. Cyanide hydratases (EC 4.2.1.66) catalysing the hydrolysis of cyanide to formamide have been purified from *G. sorghi* and *F. lateritium*, and subsequently characterized.

In this study, we report the isolation of a cyanide-degrading bacterium from the waste water of a metal-plating plant. In addition, purification and characterization of a cyanide-degrading enzyme from the strain are described.

METHODS

Isolation of the cyanide-degrading strain. The cyanide-degrading micro-organism was isolated from the waste water of a metal-plating plant by cultivation in an isolation medium containing 1 mM KCN. The isolation medium contained the following ingredients per litre distilled water: 1 g $K_2HPO_4 \cdot 2H_2O$, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.01 g $CaCl_2$, 0.01 g NaCl, 0.2 mg $MnSO_4$, 0.2 mg $CuSO_4 \cdot 5H_2O$, 0.2 mg $ZnSO_4$, 2 g glucose, 1 g tryptone. The pH of the medium was adjusted to 7.6 using HCl. KCN solution, which was sterilized using a 0.2 µm pore filter, was added to the medium before inoculation. The isolation medium in a shake flask was inoculated with the waste water and incubated at 30 °C for 48 h with shaking. The grown culture was serially diluted and plated onto TYG agar (5 g tryptone l⁻¹, 5 g yeast extract l⁻¹, 1 g glucose l⁻¹, 1 g K_2HPO_4 l⁻¹, 20 g agar l⁻¹). After 24 h incubation at 30 °C, the isolates obtained by restreaking colonies onto TYG agar were inoculated into isolation medium containing 1 mM KCN and incubated at 30 °C for 24 h. To determine cyanide-degrading activity of the isolates, the concentration of residual cyanide in each culture medium was assayed by the method of Lambert *et al.* (1975).

The cyanide-degrading bacterium was characterized by Gram-staining, quinone analysis, fatty acid profile and the API 20NE identification system (Bio Mérieux). Gram-staining was performed according to Gerhardt (1981). Fatty acids were analysed according to the method reported previously (Ikemoto *et al.*, 1978a, b; Oyaizu & Komagata, 1981) and the quinone system was determined by the method of Yamada *et al.* (1969). The API 20NE identification system was inoculated and interpreted according to the manufacturer's instructions.

Assay of cyanide-degrading activity of whole cells. The isolated strain was grown in isolation medium with or without 1 mM KCN for 24 h and harvested in the exponential growth phase by centrifugation at 4000 g for 15 min. The pellet was washed twice in 50 mM phosphate buffer (pH 7.6) and resuspended in the same buffer. A suspension of washed cells was diluted in phosphate buffer to produce a solution with an OD₆₀₀ of 1 (DU 7400 spectrophotometer, Beckman). KCN as a substrate was added to a final concentration of 1 mM in the suspension and the mixture was incubated at 30 °C. Samples were removed from the reaction mixture at appropriate intervals and the reaction was stopped by the addition of 2 vol. 1 M NaOH. The samples were then centrifuged at 10000 g and concentrations of cyanide and ammonia in the supernatants were assayed colorimetrically using the methods of Lambert *et al.* (1975) and Fawcett & Scott (1960), respectively.

Enzyme purification. The isolated strain was grown using 3 l of medium. The cells were harvested and washed as described above. The washed cell suspension was disrupted by

sonication for 10 min on ice and centrifuged at 10000 g for 15 min at 4 °C. The supernatant was used for purification of the cyanide-degrading enzyme as a cell extract. All purification steps were carried out at 4 °C. The cell extract was brought to 35% saturation with $(NH_4)_2SO_4$ by the addition of crystals. The supernatant was recovered by centrifugation and brought to 45% saturation by further addition of $(NH_4)_2SO_4$. The pellet was recovered and dissolved in 50 mM phosphate buffer (pH 7.6). This solution was applied to a DEAM column (Yamazen) previously equilibrated with 0.4 M NaCl in phosphate buffer. Proteins bound to the column were eluted with 0.5 M NaCl. An equal volume of 1.8 M $(NH_4)_2SO_4$ in phosphate buffer was added to the eluted proteins, and the mixture applied to a phenyl-Toyopearl 650 column (Tosoh) equilibrated with 0.9 M $(NH_4)_2SO_4$ in phosphate buffer. Fractions eluted with decreasing $(NH_4)_2SO_4$ concentrations of 0.54–0 M were collected. Two volumes of 1 mM phosphate buffer (pH 7.0) were added to the eluted proteins. These proteins were applied to a DEAE-Toyopearl 650 column (Tosoh) equilibrated with 0.15 M NaCl in 1 mM phosphate buffer (pH 7.0). The proteins were eluted using a NaCl gradient from 0.15 to 1 M. All active fractions were applied to a Macro-Prep ceramic hydroxyapatite column (Bio-Rad) equilibrated with 0.3 M NaCl in 1 mM phosphate buffer (pH 7.0) and eluted with a phosphate gradient of 1–250 mM. The active fractions were referred to as the purified enzyme. For gel-filtration chromatography, the purified enzyme was applied to a TSK-GEL G2000SWXL column (Tosoh), which had an exclusion limit of 100 kDa, and eluted with 50 mM phosphate buffer (pH 7.6) containing 0.3 M NaCl.

Assay of enzyme activity. Enzymic activity was assayed in 1.4 ml 50 mM phosphate buffer (pH 7.5) containing 10 mM KCN as the substrate. The reaction was carried out at 30 °C for 3–10 min. The amount of ammonia produced in the reaction mixtures was estimated using the method mentioned above. The amounts of protein were estimated using the method of Bradford (1976) with bovine serum albumin as the standard. One unit of the enzyme is defined as the amount needed to catalyse the formation of 1 µmol ammonia from cyanide min⁻¹ under the above conditions.

Other assays. The production of formate via the degradation of cyanide by the purified enzyme was assayed. The reaction was carried out in 50 mM phosphate buffer (pH 7.6) with an initial concentration of 0.1–1 mM cyanide at 30 °C. After degradation of cyanide in the reaction mixtures, concentrations of formate were determined. Formate was colorimetrically estimated by the reduction of NAD⁺ with formate dehydrogenase (Boehringer Mannheim) as described by Quayle (1966).

SDS-PAGE. SDS-PAGE was performed on 12% polyacrylamide gels according to the method of Laemmli (1970). Proteins were stained with 0.2% Coomassie brilliant blue R-250 in 50% ethanol/10% acetic acid. The molecular mass of the enzyme polypeptide was determined by comparison with the mobilities of standard proteins.

RESULTS AND DISCUSSION

Isolation and characteristics of *P. stutzeri* AK61

Several bacterial strains were isolated from the waste water of a metal plating plant with the isolation medium containing 1 mM KCN. One of them, designated AK61, exhibited cyanide-degrading activity. Strain AK61 was a

Fig. 1. Growth of *P. stutzeri* AK61 in isolation medium containing cyanide. The culture was incubated at 30 °C with shaking. The initial concentration of cyanide was 1 mM. ■, OD₆₀₀; ●, cyanide concentration in growth medium; ○, cyanide concentration in uninoculated control medium.

Gram-negative, rod-shaped bacterium that was not able to grow under anaerobic conditions. Analysis of the quinone system revealed the occurrence of a ubiquinone with nine isoprenoic units in the side chain (Q-9). The profile of cellular fatty acids obtained from strain AK61 showed high amounts of octadecanoic acid (18:0), hexadecanoic acid (16:0) and hexadecenoic acid (16:1) and the presence of 3-OH-decanoic acid (10:0-3-OH) and 3-OH-dodecanoic acid (12:0-3-OH) (data not shown). The fatty acid profile and the quinone system demonstrate that strain AK61 belongs to the genus *Pseudomonas* (Yokota *et al.*, 1992; Oyaizu & Komagata, 1983; Collins & Jones, 1981). The detection of 3-hydroxy fatty acids has been reported to be especially significant for the classification of *Pseudomonas* species (Oyaizu & Komagata, 1983). Investigation with the commercially available API 20NE identification system for strain AK61 resulted in an identification as *Pseudomonas stutzeri* with a probability of 94.2%. The DNA base composition (GC content) of strain AK61 ranged from 62 to 63 mol%, which matched with that of *P. stutzeri* (Krieg & Holt, 1984).

Degradation of cyanide by *P. stutzeri* AK61

A typical time course of cultivation of *P. stutzeri* AK61 in the isolation medium containing 1 mM KCN is shown in Fig. 1. An initial lag period of about 6 h was observed after inoculation, during which time cyanide in the medium was degraded. After this period, growth commenced. Within 17 h, all free cyanide in the solution was degraded. The final OD₆₀₀ after 30 h incubation was approximately 0.4.

A degradation profile of cyanide by the washed cells was assayed in phosphate buffer containing 1 mM KCN (Fig. 2). Rapid removal of cyanide and the simultaneous accumulation of ammonia was observed in the reaction mixtures (data not shown). The final molar yield of

Fig. 2. Degradation of cyanide by whole cells previously grown in the isolation medium with 1 mM KCN (●) and without cyanide (■). The reaction was carried out in 50 mM phosphate buffer (pH 7.6) containing 1 mM KCN at 30 °C. ○, Cyanide concentration in uninoculated control medium.

ammonia was approximately 73%. Some bacterial strains which are able to degrade cyanide and generate ammonia have been isolated. *P. fluorescens* NCIMB 11746 converted cyanide to ammonia and carbon dioxide (Dorr & Knowles, 1989). In addition, *Pseudomonas* sp. (White *et al.*, 1988), *A. xylosoxidans* subsp. *denitrificans* DF3 (Ingvorsen *et al.*, 1991) and *B. pumilus* C1 (Meyers *et al.*, 1991) were reported to convert cyanide to ammonia and formate. The cyanide-degrading activity of *P. fluorescens* NCIMB 11746 and *A. xylosoxidans* subsp. *denitrificans* DF3 was induced by cyanide during growth. On the other hand, induction of the cyanide-degrading activity of *B. pumilus* C1 was inhibited by addition of cyanide into the medium (Meyers *et al.*, 1991). The cyanide-degrading activity of *B. pumilus* C1 was observed in only late-exponential- or early-stationary-phase cells cultured without cyanide. In contrast, the cyanide-degrading activity of *P. stutzeri* AK61 was observed whether it was cultured in cyanide-containing media or not (Fig. 2). The degradation profile of cyanide by the cells cultured without cyanide was similar to that of the cells cultured with cyanide. These results suggest that the biosynthesis of the cyanide-degrading enzyme by *P. stutzeri* AK61 is independent of the presence of cyanide during culture. This property of *P. stutzeri* AK61 may be useful for applying this strain to biological treatment of cyanide and preparing the cyanide-degrading enzyme, because *P. stutzeri* AK61 does not require cyanide to produce the cyanide-degrading activity and the induction is not inhibited by cyanide.

Purification of cyanide-degrading enzyme

A cell extract of *P. stutzeri* AK61 prepared from 3 l of culture in isolation medium without cyanide showed an initial specific activity of 1.11 units (mg protein)⁻¹. The cyanide-degrading enzyme was purified by salting-out and column chromatography. The results of a typical

Table 1. Purification of cyanide-degrading enzyme from *P. stutzeri* AK61

Reactions were carried out in 1.4 ml 50 mM phosphate buffer (pH 7.5) containing 10 mM KCN for 5 min at 30 °C.

Fraction	Total protein (mg)	Total activity (units)*	Specific activity [units (mg protein) ⁻¹]	Yield (%)	Purification (-fold)
Cell extract	163.5	182	1.11	100	1.0
DEAM	1.41	47.2	33.5	26.0	30.1
DEAE-Toyopearl 650	0.78	34.5	44.2	18.9	39.7
Hydroxyapatite	0.43	23.3	54.6	12.8	49.0

* One unit of the enzyme was defined as the amount needed to catalyse the formation of 1 µmol ammonia from cyanide min⁻¹.

Fig. 3. SDS-PAGE of steps during the purification of the cyanide-degrading enzyme from *P. stutzeri* AK61. Lanes: 1 and 8, molecular mass standards (rabbit muscle phosphorylase b, BSA, hen egg-white ovalbumin, bovine carbonic anhydrase, soybean trypsin inhibitor and hen egg-white lysozyme); 2, cell extract; 3, salt-out; 4, DEAM eluate; 5, phenyl-Toyopearl eluate; 6, DEAE-Toyopearl eluate; 7, hydroxyapatite eluate.

purification are shown in Table 1. The cyanide-degrading enzyme was purified 49-fold using this procedure with an overall yield of 12.8%. The purified enzyme had a specific activity of 54.6 unit (mg protein)⁻¹. The enzyme responsible for the activity had a molecular mass of >100 kDa based on gel-filtration chromatography, but produced one major band at 38 kDa when analysed by SDS-PAGE (Fig. 3). When the NH₂-terminal amino acid sequence of the purified enzyme was determined by automated Edman degradation, only one sequence was found (Fig. 4). These results suggest that the functional enzyme is an aggregated protein that consists of a 38 kDa polypeptide subunit. The NH₂-terminal amino acid sequence of the purified enzyme had remarkable homology to those of cyanidase from *A. xylosoxidans* subsp. *denitrificans* DF3 (Ingvorsen *et al.*, 1991) and cyanide dihydratase from *B. pumilus* C1 (Meyers *et al.*, 1993). It showed a match between 16 of 25 amino acids for the NH₂-terminal amino acid

Fig. 4. NH₂-terminal amino acid sequence of the cyanide-degrading enzyme from *P. stutzeri* AK61 (AK61) and comparison with those of cyanidase from *A. xylosoxidans* subsp. *denitrificans* DF3 (DF3) and cyanide dihydratase from *B. pumilus* C1 (C1). Identical residues are enclosed by boxes.

sequence of cyanidase from *A. xylosoxidans* subsp. *denitrificans* DF3 (Fig. 4).

Kinetic studies

The initial velocity of cyanide degradation by the purified enzyme was investigated over a substrate concentration range of 0.6–10 mM. A Lineweaver–Burk plot showed a linear response over this concentration range (data not shown). A Michaelis–Menten constant (K_m) of 1.7 mM was determined for cyanide. In order to study the effect of temperature and pH on the purified enzyme activity, it was assayed over a temperature range of 15–40 °C and a pH range of 6–10. The optimum temperature for the enzyme under our assay conditions was 30 °C (data not shown), above which the activity rapidly decreased. The optimum pH for the enzymic activity was 7.5 (data not shown), above which the activity decreased; no activity was observed at pH 10.

The effects on enzyme activity of the following metal ions were investigated: K⁺, Na⁺, Fe³⁺, Ca²⁺, Mg²⁺, Zn²⁺, Mn²⁺, Ni²⁺, Hg²⁺, Cr³⁺, Co²⁺. The activity was strongly inhibited by addition of Hg²⁺ at a concentration of 0.1 mM; the relative activity was 3.6%. This suggests the possible presence of thiol groups at the catalytic site of the enzyme. The addition of the other cations to the reaction buffer did not affect the activity of the enzyme.

Fig. 5. Production of ammonia and formate after degradation of cyanide. The reactions were carried out in 50 mM phosphate buffer (pH 7.5) with various concentrations of cyanide at 30 °C for 30 min. After the cyanide was degraded, the concentrations of formate (○) and ammonia (■) in the reaction mixtures were assayed.

Relative products

As a result of cyanide degradation by whole cells, ammonia was produced in the reaction mixture. The purified enzyme was able to degrade cyanide in a phosphate buffer without a co-enzyme. In addition, the NH₂-terminal amino acid sequence of the purified enzyme shows remarkable homology to that of cyanidase from *A. xylosoxidans* subsp. *denitrificans* DF3 (Ingvorsen *et al.*, 1991). It is possible that the enzyme purified from *P. stutzeri* AK61 catalyses the same reaction as cyanidase (cyanide dihydratase), which catalyses the hydrolysis of cyanide to ammonia and formate (Ingvorsen *et al.*, 1991; Meyers *et al.*, 1993). To demonstrate that, like cyanidase, the purified enzyme converts cyanide to formate and ammonia, production of formate was assayed using formate-dehydrogenase-catalysed reduction of NAD⁺. Because cyanide forms a complex, which has an absorption maximum at approximately 320 nm, with NAD⁺ (Imai & Suzuki, 1970), ammonia and formate concentrations were estimated after the cyanide in the reaction mixtures was completely degraded. The purified enzyme was added to phosphate buffer solutions containing several different concentrations of cyanide, and the final concentrations of formate and ammonia in the reaction mixtures were estimated. The final concentrations of formate and ammonia increased linearly, and the amounts of the products were equivalent to approximately 80% of the initial concentration of cyanide (Fig. 5). This result showed that the enzyme from *P. stutzeri* AK61 catalysed the hydrolysis of cyanide like cyanidase and that *P. stutzeri* AK61 degrades cyanide by the same pathway as *A. xylosoxidans* subsp. *denitrificans* DF3 and *B. pumilus* C1.

Cyanidase from *P. stutzeri* AK61 has similar properties to cyanidase from *A. xylosoxidans* subsp. *denitrificans* DF3 (Ingvorsen *et al.*, 1991) and cyanide dihydratase from *B. pumilus* C1 (Meyers *et al.*, 1991). These enzymes had molecular masses of over 100 kDa based on gel-

filtration chromatography, and the polypeptide chains of the enzymes had molecular masses of approximately 38 kDa as shown by SDS-PAGE. In addition, the enzymes had a *K_m* of approximately 2 mM for cyanide and the NH₂-terminal amino acid sequence of cyanidase from *P. stutzeri* AK61 shows a remarkable homology to that of cyanidase from *A. xylosoxidans* subsp. *denitrificans* DF3 (Ingvorsen *et al.*, 1991). These enzymes, however, also show a number of different properties. The three enzymes show different optimum temperatures (26 °C for cyanidase from *A. xylosoxidans* subsp. *denitrificans* DF3, 37 °C for cyanide dihydratase from *B. pumilus* C1 and 30 °C for the purified enzyme in this paper). The activity of cyanide dihydratase from *B. pumilus* C1 was significantly enhanced by addition of Cr³⁺ at a concentration of 5–500 μM (Meyers *et al.*, 1991). However, the addition of 100 μM Cr³⁺ did not affect the activity of cyanidase from *P. stutzeri* AK61. The results of SDS-PAGE, gel-filtration chromatography and the NH₂-terminal amino acid sequence of cyanidase from *P. stutzeri* AK61 showed that this enzyme consists of one kind of polypeptide. In contrast, cyanidase (cyanide dihydratase) from *A. xylosoxidans* subsp. *denitrificans* DF3 (Ingvorsen *et al.*, 1991) and *B. pumilus* C1 (Meyers *et al.*, 1993) consisted of two and three kinds of polypeptide chains, respectively. Proteins which consist of one polypeptide are suitable for gene cloning and for expression in an active form.

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