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An enzymatic method for determination of azide and cyanide in aqueous phase

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ABSTRACT

A halohydrin dehalogenase (HHDH-PL) from *Parvibaculum lavamentivorans* DS-1 was characterized and applied to determine azide and cyanide in the water. In this methodology, HHDH-PL catalysed azide and cyanide to react with butylene oxide and form corresponding β-substituted alcohols 1-azidobutan-2-ol (ABO) and 3-hydroxypentanenitrile (HPN) that could be quantitatively detected by gas chromatograph. The detection calibration curves for azide (R^2 = 0.997) and cyanide (R^2 = 0.995) were linear and the lower limits of detection for azide and cyanide were 0.1 and 0.3 mM, respectively. Several other nucleophiles were identified having no effect on the analysis of azide and cyanide, excepting nitrite which influenced the detection of cyanide. This was the first report of a biological method to determine the inorganic azide and cyanide by converting them to the measurable organics.

1. Introduction

Many toxic compounds are produced from chemistry synthesis and other manufacturing industries. Although the new concept of "green chemistry" concerns the development of methods using nontoxic chemicals and smaller amounts of reagent (Lozowski, 2014; Ritter, 2014), some synthetic routes involving toxic reagent are inescapable, such as azide-mediated azidolysis and cyanide-mediated cyanation processes. Azide and cyanide are used as important nucleophiles and widely applied to the synthesis of fine chemicals (Fotsing and Banert, 2005; Han et al., 2014; Intrieri et al., 2014; Powell et al., 2014; Xie et al., 2014; Yin et al., 2014). However they are highly toxic substances that inhibit cytochrome oxidase, which are harmful to human health and ecological environment. Hence, the contents of azide and cyanide in the water should be strictly monitored and controlled. Currently, a high throughput and environmental-friendly analytical procedure is in demand for analytical methods. Many successful direct or indirect analytical methods, including gas chromatograph (GC), GC mass spectrometry, spectrophotometry, high efficiency liquid chromatography, and ion chromatography have been reported to determine the azide (Gennaro et al., 1993; Kage et al., 2000; Meatherall and Palatnick, 2009; Minakata et al., 2012) and cyanide (Agrawal et al., 2005; Chadha and Lawrence, 1991; Mousavi et al., 2013; Vallejo-Pecharroman and de Castro, 2002).

Halohydrin dehalogenase (HHDH, EC4.5.1.X) (de Jong et al., 2005; Vlieg et al., 2001 You et al., 2013), a promiscuous enzyme from short chain dehydrogenase/reductase enzyme family, is able to catalyse the dehalogenation of vicinal haloalcohols and ring-opening of epoxides by many nucleophiles (Fig. 1) (Hasnaoui-Dijoux et al., 2008; Janssen et al., 2006; Spelberg et al., 2001). HHDHs exhibit high enantioselectivity and regioselectivity in kinetic resolution of β-halohydrins and epoxides, and serve as the attractive biocatalysts in the preparation of optically active pharmaceutical intermediates and fine chemicals (Campbell-Verduyn et al., 2010; Elenkov et al., 2012; Haak et al., 2007; Ma et al., 2010). For the last few years, several HHDHs were cloned and engineered by our group for manufacturing high value epoxides and β-substituted alcohols (Jin et al., 2012; Liu et al., 2014; Xue et al., 2014). Recently, a novel HHDH (HHDH-PL) from *P. lavamentivorans* DS-1 has been cloned and characterized, which exhibits high activity to many aliphatic and aromatic haloalcohols (Wan et al., 2014). HHDH-PL also shows high azidolysis and cyanation activities toward many epoxides, which make it possible applications in the determination of the azide and cyanide by quantitative analysis of the formed β-substituted alcohols.

In this research, a sensitive and simple method was developed to determine azide and cyanide in the water. HHDH-PL was used as a biocatalyst to convert azide and cyanide into β-substituted

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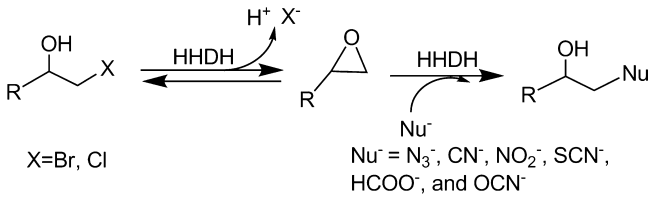

Fig. 1. Summary of HHDH-catalysed ring-closure and ring-opening reactions.

alcohols (ABO and HPN) by ring-opening of butylene oxide. And then azide and cyanide could be indirectly detected by GC analysis of the formed ABO and HPN (Fig. 2).

2. Materials and methods

2.1. Materials and reagents

Butylene oxide, 1,3-dichloro-2-propanol (1,3-DCP) and epichlorohydrin (ECH) were purchased from Sigma–Aldrich (Shanghai, China). Sodium cyanide was supplied by Zhejiang Neo-Dankong Pharmaceutical Co., Ltd. (Taizhou, China). Analytical reagent sodium azide, sodium thiocyanate, sodium cyanate, sodium nitrite, sodium thiocyanate, sodium chloride, sodium bromide and other regents were from Aladdin (Shanghai, China). ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AVANCE III (1H 500 MHz) in $CDCl_3$. Chemical shift (δ) was given in ppm downfield from TMS as the internal standard. Recombinant HHDH-PL was expressed in *Escherichia. coli* BL (DE3), and purified by Nickel-NTA column (Fig. S1).

2.2. Analytical methods

GC determination of 1,3-DCP and ECH was performed on Agilent GC 7890A equipped with FID detector and HP-5 column (Agilent). Nitrogen was used as carrier gas at a flow rate of 1.0 mL/min. The inlet and detector temperatures were set at 230 and 250 °C, respectively. The temperature program was isothermal at 60 °C for 4 min, increase at 20 °C/min to 120 °C. The retention times of 1,3-DCP, ECH and butylene oxide were 4.04, 6.41 and 2.71 min, respectively.

GC assay of ABO and HPN were performed on GC-14C (Shimadzu, Japan) equipped with FID detector and the chiral capillary the Astec CHIRALDEX™ G-TA column (30 m × 0.25 mm, 0.12 μm film thickness, Supelco, USA). Helium was used as carrier gas at a flow rate of 1.0 mL/min. The inlet and detector temperatures were all set at 220 °C. The split ratio was 30:1 and injection volume was 1 μL. The temperature program was isothermal at 120 °C for 5 min, increase at 5 °C/min to 140 °C, final 2 min at 140 °C. Retention times for ABO and HPN were 4.62 and 7.56 min respectively.

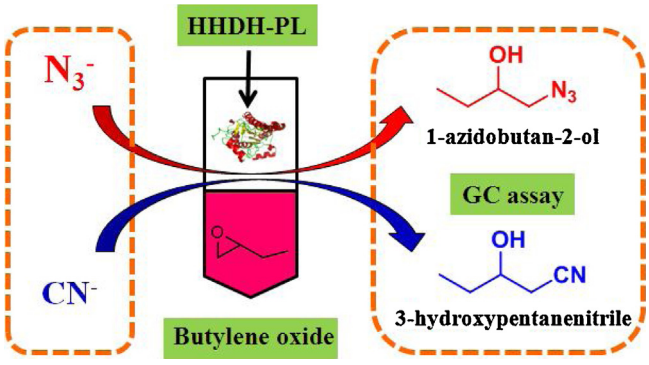

Fig. 2. Overview of the method for determining azide and cyanide.

2.3. pH and temperature dependences of HHDH-PL

Standard halohydrin substrate 1,3-DCP was used to test the pH and temperature dependences of HHDH-PL. Enzyme activities were calculated using the yield of ECH. The optimal pH was assayed in 200 mM buffers: HAc–NaAc (pH 4.0–6.0), Na_2HPO_4–NaH_2PO_4 (pH 6.0–8.0), Tris–H_2SO_4 (pH 7.0–9.0) and Gly–NaOH (pH 8.5–10.0). The pH stability of HHDH-PL was examined by incubating the enzyme in different pH buffers (ranging from 6.0 to 10.0) at 4 °C for 2 h. Residual activities of the treated enzymes were analysed in PBS buffer (pH 7.5, 200 mM). The optimal temperature was determined at the temperature ranging from 25 to 70 °C. The thermal stability study was performed by incubating the enzyme at 30, 40, 50 and 60 °C. The residual activities were monitored by periodically taking samples from the treated enzymes. The standard activity assay procedure: a 2 mL tube containing 20 mM 1,3-DCP, 20 μL (1.6 μg/μL) purified HHDH-PL and the buffers (the final volume was 1 mL) was stirred on Thermomixer comfort (Eppendorf, Hamburg, Germany) for 10 min, and the activity was assayed by extracting with 1 mL ethyl acetate.

2.4. Effects of metal ions and chemicals on HHDH-PL activity

The influences of metal ions, EDTA, Tween 20 and Tween 80 on HHDH-PL activity were tested. The reactions were carried out at 40 °C in PBS buffer (pH 7.5, 200 mM) by adding chemicals. Initial activities were analysed after 10 min and compared to the activity without treatment by chemicals.

2.5. Synthesis of ABO and HPN

ABO and HPN were prepared using whole-cell biocatalysis of HHDH-PL. Reactions were carried out in a 250 mL reactor containing 100 mL PBS (pH 7.5, 200 mM), 1 mL butylene oxide, 2 g sodium azide or 1.5 g sodium cyanide and 8 g (wet cells) HHDH-PL. The processes were monitored by GC and stopped at the 100% conversion of butylene oxide. Cells were removed by centrifugation at 9000 × *g* for 10 min. The supernatants were extracted with ethyl acetate (3 × 100 mL). The organics were combined and dried on anhydrous sodium sulfate. After removing ethyl acetate under vacuum, the yellow liquids of ABO (85% yield) and HPN (79% yield) were obtained respectively. NMR characterization of ABO and HPN were performed (Fig. S2–S5). ABO: ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.71–3.37 (dd, J = 5.8, 3.3 Hz, 1H), 3.29 (dd, J = 12.4, 3.3 Hz, 1H), 3.27 3.25 (dd, J = 12.4, 7.4 Hz, 1H), 2.05 (s, 1H), 1.55 1.52 (m, 2H), 1.00 0.97 (t, J = 7.5 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 72.28, 56.71, 56.71, 27.30, 9.63. HPN: ^{1}H NMR (500 MHz, CDCl3) δ 3.89–3.86 (dd, J = 6.1, 5.0 Hz, 1H), 2.56–2.47 (m, 3H), 1.64–1.61 (m, 2H), 1.00–0.97 (t, J = 7.5 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 117.92, 69.14, 29.28, 25.57, 9.90.

2.6. Determination of calibration curves of azide and cyanide

The standard concentrations of sodium azide (ranging from 0.2 to 2.0 mM), sodium cyanide (ranging from 0.6 to 4.0 mM) and butylene oxide (100 mM) were prepared using PBS buffer as solvent. 500 μL sodium azide or sodium cyanide solution, 450 μL butylene oxide solution and 50 μL HHDH-PL were mixed in a 2 mL tube and stirred on Thermomixer for 30 min. The yields of ABO and HPN were determined by GC by extracting the samples with 1 mL ethyl acetate. On the other hand, standard concentration of ABO (ranging from 0.1 to 1.0 mM) and HPN (ranging from 0.3 to 2.0 mM) were also prepared and analysed by GC.