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Asymmetric reduction of 2-chloroacrylic acid to (*S*)-2-chloropropionic acid by a novel reductase from *Burkholderia* sp. WS

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Abstract—*Burkholderia* sp. WS has a novel enzyme that catalyzes the asymmetric reduction of 2-chloroacrylic acid to yield (*S*)-2-chloropropionic acid, which is used as a building block for the synthesis of aryloxyphenoxypropionic acid herbicides. NADPH is required as a co-substrate for this reaction.

1. Introduction

Biologically active compounds, including pharmaceutical and agrochemical products, often contain stereogenic centers. In most cases, one of the enantiomers has the desired biological activity, whereas the other does not or is even hazardous. Thus, there exists a continuing demand for the development of technology to produce optically active compounds efficiently.

(*S*)-2-Chloropropionic acid (CPA) is used as a building block for the synthesis of aryloxyphenoxypropionic acid, the (*R*)-isomer of which has herbicidal activity. In the industrial synthesis of this herbicide, (*S*)-2-CPA is produced by resolution, in which the (*R*)-2-CPA component of a racemic 2-CPA mixture is selectively degraded using (*R*)-2-haloacid dehalogenase.[1] Since the theoretical maximum yield of (*S*)-2-CPA from a racemic mixture of 2-CPA by this procedure is only 50%, it would be advantageous to establish an asymmetric synthesis procedure that could produce (*S*)-2-CPA directly. Herein, we have developed an enzymatic process in which (*S*)-2-CPA is produced by asymmetric reduction of the carbon–carbon double bond in 2-chloroacrylic acid (2-CAA).

2. Results and discussion

We have previously isolated bacterial strains that use 2-CAA as their sole carbon and energy source.[2] One of these strains, formerly called *Pseudomonas* sp. WS, was used herein. We re-characterized this strain by 16S rRNA sequencing and found that the sequence had the highest similarity to those of bacteria belonging to the genus *Burkholderia*. Thus, we have renamed this strain *Burkholderia* sp. WS.

Burkholderia sp. WS exhibits 2-CPA dehalogenase activity when grown in media containing 2-CPA as the sole carbon source.[2] We also found that this bacterium has a gene homologous to the (*S*)-2-haloacid dehalogenase [(*S*)-DEX] gene (data not shown). The deduced amino acid sequence of (*S*)-DEX of *Burkholderia* sp. WS [(*S*)-DEX WS] is highly similar to that of *Pseudomonas* sp. YL (76% identity),[3] which catalyzes the hydrolysis of (*S*)-2-CPA with inversion of the C_2 configuration to produce (*R*)-lactate.[4–6] Although 2-CPA dehalogenase activity was too low to be detected in a crude extract of cells grown on 2-CAA,[2] the above-mentioned observations raised the possibility that 2-CAA is first converted into (*S*)-2-CPA, and that (*S*)-2-CPA is subsequently hydrolyzed to produce (*R*)-lactate by (*S*)-DEX WS (Scheme 1).

In order to verify this scheme, we first examined whether intact *Burkholderia* sp. WS cells can metabolize 2-CAA to release chloride ions. Cells were grown in the

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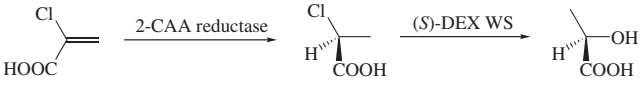
following medium: 0.2% 2-CAA, 0.2% $(NH_4)_2SO_4$, 0.05% yeast extract (DIFCO), 0.1% K_2HPO_4, 0.2% NaH_2PO_4, and 0.01% $MgSO_4{\cdot}7H_2O$, pH 7.1. The reaction was carried out at 30 °C in a mixture consisting of 60 mM potassium phosphate buffer (pH 7.1), 0.2% 2-CAA, and 1% (w/v) cells. We monitored the reaction by measuring the concentrations of 2-CAA and the chloride ions. 2-CAA was analyzed by high-performance liquid chromatography (HPLC) using an Asahipak ODP-50 column (4.6 mm by 25 m, Showa Denko) eluted using 20% acetonitrile containing 0.1% trifluoroacetic acid at a flow rate of 0.5 mL/min and temperature of 25 °C. Eluted products were detected at 246 nm. Chloride ions were quantified spectrophotometrically using mercuric thiocyanate and ferric ammonium sulfate.[7] As shown in Figure 1, the concentration of chloride ions increased as the 2-CAA concentration decreased. This result is consistent with Scheme 1, although it does not exclude the possibility that the chloride ion was released directly from 2-CAA.



Scheme 1. Putative pathway of 2-CAA metabolism in *Burkholderia* sp. WS.

In order to see whether 2-CPA was produced as a metabolite of 2-CAA, the culture medium was analyzed by gas chromatography–mass spectrometry (GC–MS) (Fig. 2). The culture supernatant (1 mL) was dried and the residue was solubilized in 0.2 mL of methanol. The sample was injected into a GC–MS system equipped with a capillary column (DB-WAX, 0.32 mm by 30 m, J & W). The temperature was kept at 50 °C for 1 min and then raised to 180 °C at a rate of 15 °C/min. Mass spectrometry was conducted in the EI^+ ionization mode with a mass range of 29–130 *m*/*z*. The culture medium was shown to contain a metabolite eluting at a retention time of 12.6 min (Fig. 2c), which is identical to that of authentic 2-CPA (Fig. 2a). The mass spectrum of the metabolite shows peaks at *m*/*z* values of 35 and 45, which were assigned to the chloride atom and the carboxyl group, respectively (Fig. 2d). The fragment ion at an *m*/*z* of 64 was probably produced by the release of COO (*m*/*z* = 44) from [^{35}Cl]-2-CPA (*m*/*z* = 108). All of these peaks were found in the spectrum of authentic 2-CPA (Fig. 2b). Thus, although the unstable molecular ion of [^{35}Cl]-2-CPA (*m*/*z* = 108) was not detected in the mass spectrum (Fig. 2d), the metabolite in the culture medium could be identified as 2-CPA.

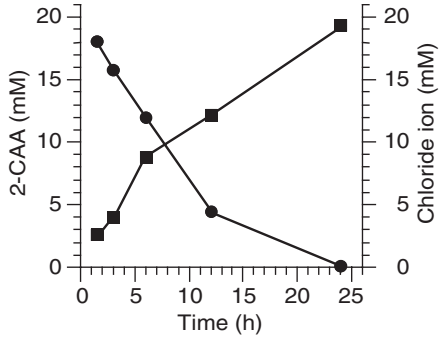


Figure 1. Degradation of 2-CAA and production of chloride ions using *Burkholderia* sp. WS cells. The concentrations of 2-CAA (●) and chloride ion (■) in the reaction mixture were monitored.

Next, GC using a chiral column was used to determine the configuration of the 2-CPA, that was generated. First, the 2-CPA produced by the *Burkholderia* sp. WS cells was treated with a boron trifluoride methanol solu-

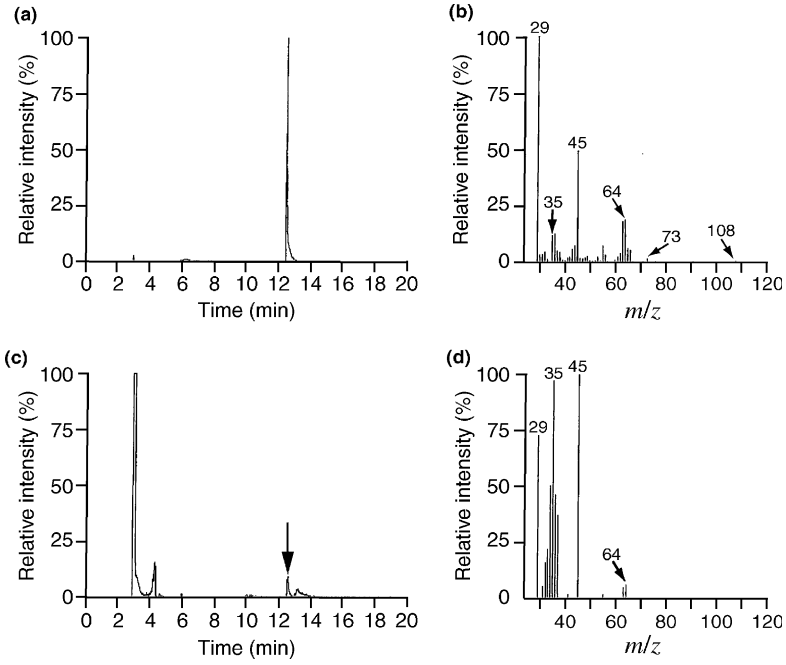


Figure 2. GC–MS analysis of the metabolite of 2-CAA. (a) Chromatogram of authentic 2-CPA. (b) Mass spectrum of authentic 2-CPA. (c) Chromatogram of the metabolite. (d) Mass spectrum of the compound indicated by the arrow in (c).

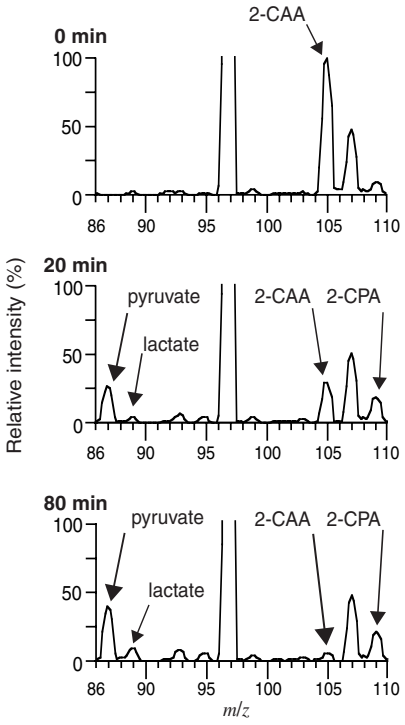


Figure 3. Mass spectrometric monitoring of the degradation of 2-CAA using a cell-free extract in the presence of NADPH. Spectra were obtained for the range between 86 and 110 atomic mass units. [^{35}Cl]-2-CAA, [^{37}Cl]-(*S*)-2-CPA, lactate, and pyruvate were detected at *m*/*z* values of 105, 109, 89, and 87, respectively.

tion to convert it into a methyl ester, extracted with ethyl acetate, and concentrated. GC analysis of the 2-CPA methyl ester was performed on a GC-14A chromatograph (Shimadzu) equipped with a flame ionization detector and a chiral chromatographic column (CP-Chirasil-DEX CB, 0.32 mm by 25 m, Varian). The column temperature was kept at 70 °C. The 2-CPA methyl ester sample derived from the product of the *Burkholderia* sp. WS cells exhibited a single peak and co-eluted with authentic (*S*)-2-CPA methyl ester, and not with authentic (*R*)-2-CPA methyl ester (data not shown). These results demonstrate that *Burkholderia* sp. WS cells produce the (*S*)-enantiomer of 2-CPA, using an enzyme that catalyzes the asymmetric reduction of 2-CAA to (*S*)-2-CPA.

Next, using a cell-free extract of *Burkholderia* sp. WS, we identified the co-substrate required for this reaction. We incubated at 30 °C for 2 h a reaction mixture containing 60 mM ammonium acetate buffer (pH 7.1), 5 mM 2-CAA, 5 mM co-substrate, and the cell-free extract (1.1 mg protein/mL). The following compounds were tested as potential co-substrates: NADH, NADPH, reduced FAD, reduced FMN, sodium dithionite, benzyl viologen, and sodium borohydride. The reaction was terminated by the addition of acetonitrile. The reaction mixture was analyzed by electrospray ionization mass spectrometry (ESI-MS) using an API3000 LC/MS/MS system (PE Sciex) in the negative-ion mode. We found that the cell-free extract catalyzed the reduction of 2-CAA only in the presence of NADPH (Fig. 3). Over the course of the reaction, the peak corresponding to [^{35}Cl]-2-CAA at $m/z = 105$ decreased, while the peaks corresponding to [^{37}Cl]-2-CPA ($m/z = 109$) and lactate ($m/z = 89$) increased in size. These trends support the metabolic pathway shown in Scheme 1. In this experiment, an increase in size of the peak at $m/z = 87$ was also observed, suggesting that lactate is further converted into pyruvate.

3. Conclusion

In summary, we found that 2-CAA-grown *Burkholderia* sp. WS produces a novel enzyme that catalyzes the asymmetric reduction of 2-CAA to (*S*)-2-CPA. This enzyme required NADPH as a co-substrate. Since (*S*)-2-CPA is probably further metabolized by (*S*)-DEX, deletion of the (*S*)-DEX gene would be essential in order to accumulate (*S*)-2-CPA in *Burkholderia* sp. WS. Cloning of the gene encoding this novel enzyme, as well as the overproduction and further characterization of the gene product, are currently in progress. It may be possible to produce a large amount of (*S*)-2-CPA by coupling this 2-CAA reducing enzyme with a system for regenerating NADPH.

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