

# Enzymatic Dehalogenation of Chlorinated Nitroaromatic Compounds

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**4-Chlorobenzoate dehalogenase from *Pseudomonas* sp. strain CBS3 converted 4-chloro-3,5-dinitrobenzoate to 3,5-dinitro-4-hydroxybenzoate and 1-chloro-2,4-dinitrobenzene to 2,4-dinitrophenol. The activities were 0.13 mU/mg of protein for 4-chloro-3,5-dinitrobenzoate and 0.16 mU/mg of protein for 1-chloro-2,4-dinitrobenzene compared with 0.5 mU/mg of protein for 4-chlorobenzoate.**

Nitroaromatic compounds are widely used in the synthesis of pesticides, drugs, dyes, and explosives (3, 16). Some of these compounds carry not only a nitro group, but also a chlorine substituent on the aromatic ring (10, 11). A large number of chlorinated nitroaromatic compounds are intermediates in the synthesis of chlorinated anilines. During the synthesis of such products, side products very often are formed which carry more than the desired nitro or chloro-group. These higher chlorinated or nitrated compounds usually end up in the wastewater of production plants, where they cause serious problems (1). Many of these compounds are highly toxic to bacteria. Oxygenases which usually initiate the degradation of other aromatic compounds such as benzene or toluene by an electrophilic attack cannot attack these compounds since the electron density at the aromatic ring in these compounds is very low. Thus, other reactions that proceed via a nucleophilic attack at the aromatic ring have to be considered for the degradation of these substances. In previous papers we reported the replacement of a chlorine substituent from 4-chlorobenzoate by a hydroxy group derived from water catalyzed by an enzyme from *Pseudomonas* sp. strain CBS3 (9, 14). Assuming that this reaction proceeds through a nucleophilic attack, the replacement of a chlorine in chloroaromatics carrying a nitro group in the *ortho*- or *para*-position should be facilitated. Such a replacement would lead to nitrophenols, whose microbial degradation is well understood (3, 10, 13, 16, 17). We therefore checked the activity of 4-chlorobenzoate dehalogenase from *Pseudomonas* sp. strain CBS3 towards different nitroaromatic compounds.

## MATERIALS AND METHODS

**Bacteria.** *Pseudomonas* sp. strain CBS3 was originally isolated from soil by using 4-chlorobenzoate as sole source of carbon and energy (5). The bacteria were grown overnight in a 100-liter fermentor inoculated with 1 liter of an exponentially growing culture. The medium contained the following, per liter:  $K_2HPO_4$ , 2 g;  $KH_2PO_4$ , 1 g;  $MgSO_4 \cdot 7H_2O$ , 0.2 g;  $NH_4Cl$ , 2 g; yeast extract, 0.2 g; 4-chlorobenzoate, 0.5 g. Cells were harvested by centrifugation and stored at  $-20^\circ C$  until used. The average yield was 35 g (wet weight) of cells per fermentor.

**Preparation of cell-free extracts.** A 50-g portion of the frozen cells was suspended in 50 ml of 50 mM potassium phosphate buffer, pH 7, using a Braun homogenizer. The bacteria were disrupted by two passes through a French

pressure cell at 130 MPa. Cell debris was removed by centrifugation at  $40,000 \times g$  for 30 min.

**Enrichment of 4-chlorobenzoate dehalogenase by ammonium sulfate precipitation.** Solid ammonium sulfate was added to the crude extract to 30% saturation. The resulting precipitate was removed by centrifugation. Ammonium sulfate was added to the supernatant to a final concentration of 60%. The precipitate was collected by centrifugation and redissolved in 50 mM potassium phosphate buffer, pH 7. This solution was then dialyzed overnight against 5 liters of the same buffer.

**Preparation of 4-chlorobenzoate dehalogenase by  $MgCl_2$  extraction.** An alternative method used for preparation of 4-chlorobenzoate dehalogenase was  $MgCl_2$  extraction of whole cells of *Pseudomonas* sp. strain CBS3. A 30-g portion of deep-frozen cells was suspended in 20 ml of 0.1 M Tris hydrochloride buffer, pH 8.4, containing 0.2 M  $MgCl_2$ . After 1 h at  $4^\circ C$ , cells were removed by centrifugation at  $45,000 \times g$ . The supernatant contained the 4-chlorobenzoate dehalogenase. Photometric assays were performed with this enzyme preparation.

**Protein determination.** In crude extracts, protein was determined by the biuret method (6); for enriched fractions, the Bradford assay (2) was used, with bovine serum albumin as standard.

**Enzyme assays.** 4-Chlorobenzoate dehalogenase activity with 4-chlorobenzoate as substrate was tested as described before (14). For activity with the nitro compounds, 880  $\mu l$  of enzyme was preincubated for 10 min with 20  $\mu l$  of 0.1 M  $MnCl_2$ . Then 100  $\mu l$  of a 100 mM substrate solution in methanol was added. Spectra of the solutions were recorded every 5 min from 500 to 300 nm. The reference cuvette contained the enzyme with  $MnCl_2$  and with methanol instead of the substrate solution. As a control, substrates were also incubated with buffer without enzyme and the spectra were recorded as described above. Enzyme activity was calculated from the increase at the absorbance maxima of the reaction products ( $\lambda_{max} = 425$  nm,  $\epsilon = 7,200 M^{-1} cm^{-1}$  for 3,5-dinitro-4-hydroxybenzoate; and  $\lambda_{max} = 354$  nm,  $\epsilon = 12,000 M^{-1} cm^{-1}$  for 2,4-dinitrophenol).

**Identification of reaction products.** A 1-mg amount of 4-chloro-3,5-dinitrobenzoate and 1 mg of 1-chloro-2,4-dinitrobenzene were incubated overnight with 30 ml of the ammonium sulfate-fractionated 4-chlorobenzoate dehalogenase (0.3 mU/ml). As controls, the substrates were incubated in buffer without enzyme and the enzyme was incubated without substrate. The reaction was stopped by the addition of 2 ml of 6 N HCl. Then the solutions were extracted twice with 5 ml of ethylacetate. A sample of the ethyl acetate layer

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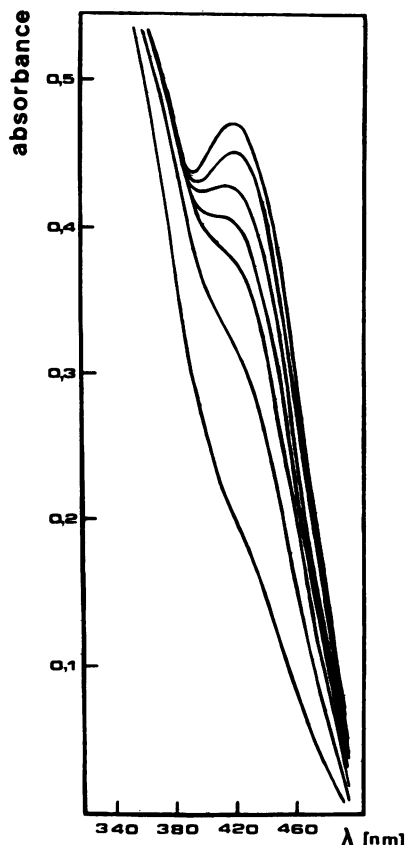


FIG. 1. Spectral changes during reaction of 4-chlorobenzoate dehalogenase with 4-chloro-3,5-dinitrobenzoate. The assay contained 880  $\mu$ l of enzyme solution, 20  $\mu$ l of 0.1 M  $\text{MnCl}_2$ , and 100  $\mu$ l of 10 mM substrate in methanol. To the reference was added 100  $\mu$ l of methanol without substrate. The temperature was 30°C, and the spectra were recorded every 5 min from 500 to 350 nm.

was applied to thin-layer chromatography plates, which were developed with toluene-ethyl acetate-acetic acid (60:30:5, vol/vol/vol). The ethyl acetate layer was evaporated to dryness, and diazomethane in ether was added to the residue to methylate acidic OH groups. After removal of the ether, the residue was dissolved in 50  $\mu$ l of methanol. A 0.1- $\mu$ l portion of this solution was injected onto a capillary gas chromatography column (SE30; 25 m; 80 to 240°C, 10°C/min; injector temperature, 250°C), which was connected to a mass spectrometer (Varian 3700). Mass spectra of each emerging peak were recorded.

## RESULTS

**Dehalogenation of 4-chloro-3,5-dinitrobenzoate.** When 4-chlorobenzoate dehalogenase from *Pseudomonas* sp. strain CBS3 was incubated with 4-chloro-3,5-dinitrobenzoic acid, the solution turned yellow. Figure 1 shows the spectral changes observed in such an assay. The maximum increase corresponded to the absorbance maximum of 3,5-dinitro-4-hydroxybenzoic acid at 425 nm. In controls without enzyme, the spectra did not change. In thin-layer chromatography, a new yellow spot appeared which comigrated with authentic 3,5-dinitro-4-hydroxybenzoic acid. After methylation, a new peak was observed in the gas chromatogram, which corresponded in retention time (9 min 11 s for the standard and 9 min 17 s for the metabolite) and in mass spectrum to authentic 3,5-dinitro-4-hydroxybenzoic acid methylester (Fig. 2). At higher concentrations of diazomethane in the methylation reaction, a second new peak appeared at 9 min 30 s with a mass spectrum corresponding to that of standard 3,4-dinitro-4-methoxybenzoic acid methylester. Thus, 4-chlorobenzoate dehalogenase from *Pseudomonas* sp. strain CBS3 seems to dehalogenate 4-chloro-3,5-dinitrobenzoic acid the same way it dehalogenates 4-chlorobenzoate. Activity with the nitro compound was 0.13 mU/mg of protein versus 0.5 mU/mg of protein for 4-chlorobenzoate. Due to the high extinction coefficient of the product, the conversion

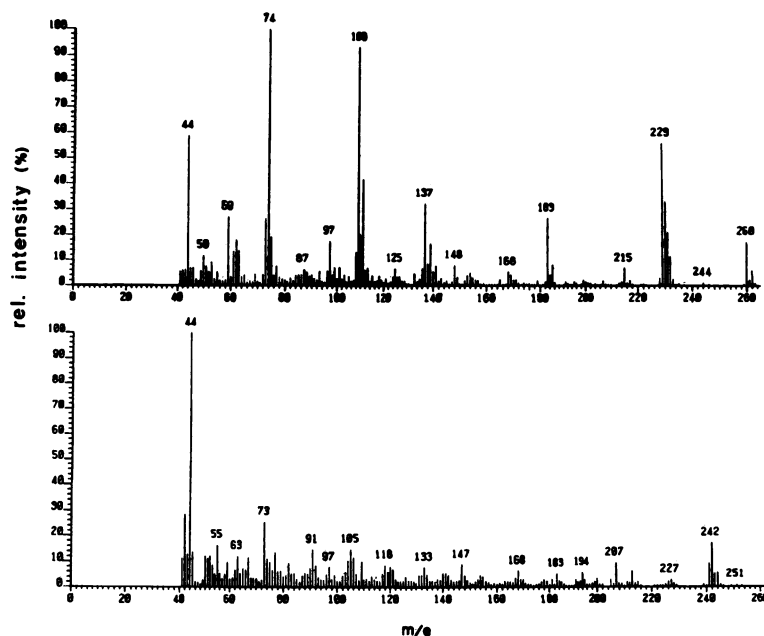


FIG. 2. Mass spectra of the methylated substrate 4-chloro-3,5-dinitrobenzoic acid methylester (top) and the methylated product 4-hydroxy-3,5-dinitrobenzoic acid methylester (bottom). For conditions, see Materials and Methods.

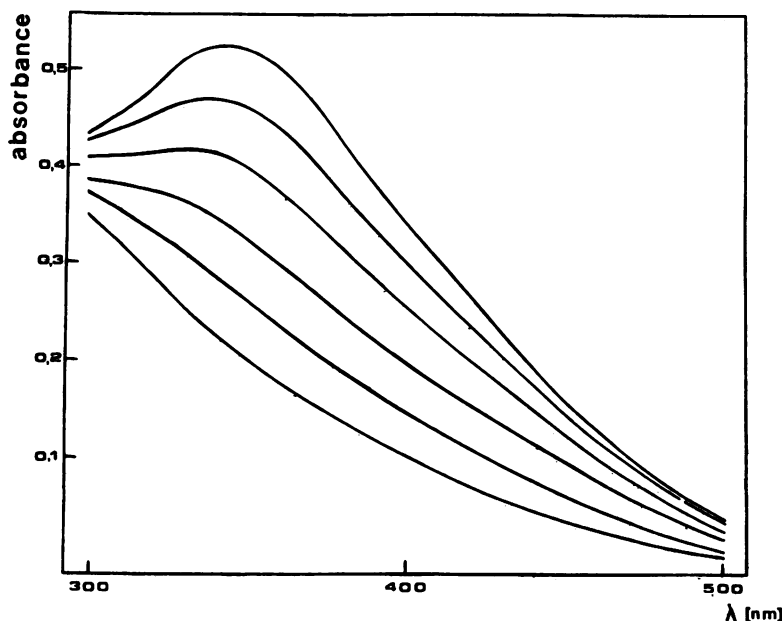


FIG. 3. Spectral changes during the reaction of 4-chlorobenzoate dehalogenase with 1-chloro-2,4-dinitrobenzene as substrate. The assay was the same as in the legend to Fig. 1. The spectra were recorded every 20 min from 500 to 300 nm.

of this nitro compound can be used as a convenient and sensitive test for the enzyme.

**Dehalogenation of 1-chloro-2,4-dinitrobenzene.** So far the hydrolytic dehalogenation of aromatic compounds has only been shown with 4-halogenated benzoates. The carboxy group in the *para*-position of the halogen seemed to be essential for the enzyme. Even 4-chlorobenzoate methyl-ester was not dehalogenated (14a). We assumed that the reaction of 4-chlorobenzoate dehalogenase proceeds via a nucleophilic substitution of the chlorine by the hydroxy group. To check whether the carboxy group is also essential when the aromatic ring is activated for nucleophilic substitution by two nitro substituents, we used 1-chloro-2,4-dinitrobenzene as substrate. When 1-chloro-2,4-dinitrobenzene was incubated with 4-chlorobenzoate dehalogenase, a pale yellow color developed. The maximum increase in the absorbance spectrum (Fig. 3) corresponded to the absorbance maximum of 2,4-dinitrophenol ( $\lambda_{\max} = 354$  nm). The gas chromatogram after methylation of the ethyl acetate extract of the reaction mixture showed one new peak which corresponded in retention time (11 min 26 s for the standard and 11 min 17 s for the metabolite) and in mass spectrum to those of authentic 2,4-dinitro-1-methoxybenzene (Fig. 4). In controls without enzyme, no phenol was found. The activity with 1-chloro-2,4-dinitrobenzene was 0.16 mU/mg of protein compared with 0.5 mU/mg for 4-chlorobenzoate. These results show that 4-chlorobenzoate dehalogenase can dehalogenate 1-chloro-2,4-dinitrobenzene, although this substance does not carry a carboxy group.

## DISCUSSION

In nitroaromatic compounds, the aromatic ring has a very low electron density. Therefore, these substances are resistant to electrophilic attack by dioxygenases, which is the first step in the oxidative degradation of many aromatic compounds. It is therefore difficult to isolate bacteria able to degrade these compounds. Nevertheless, several publications deal with the microbial metabolism of nitroaromatics.

Degradation of such compounds can occur in two ways. First, the nitro group can be reduced to an amino group. The resulting aniline derivatives can then be degraded via oxidation by dioxygenases (7). The second way is by oxidative removal of the nitro group. Phenols and nitrite are the products (10, 13, 16, 17). Enzymes have been described for

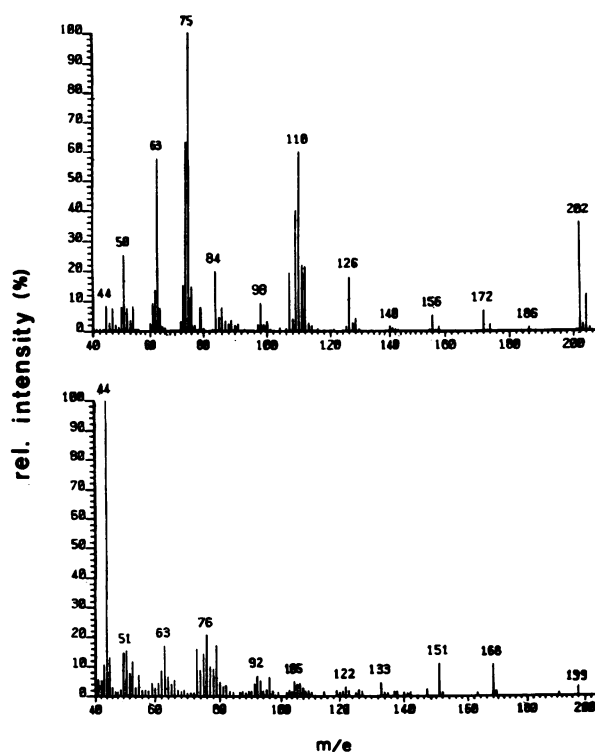


FIG. 4. Mass spectra of the substrate 1-chloro-2,4-dinitrobenzene (top) and the methylated product 2,4-dinitro-1-methoxybenzene (bottom). For conditions, see Materials and Methods.

both cases (8, 12, 16, 17). For chlorinated aromatic compounds, again, two methods of degradation exist. The aromatic ring can be oxidized to the catechol, which is then cleaved between the hydroxy groups, and the chlorine is removed in a later step (4), or the chlorine can be removed in the first step as in the case of 4-chlorobenzoate. Very little is known about the degradation of aromatic compounds carrying a nitro and a chloro group. Generally, these compounds are considered recalcitrant. It was shown that pentachloronitrobenzene, however, can be denitrated reductively to pentachlorobenzene in a glutathione-dependent reaction in rats (11), and with the herbicide Chlornitrofen (4-nitrophenyl-2,4,6-trichlorophenylether) a reductive dehalogenation in soil was observed (15).

From the data presented here, it is evident that 4-chlorobenzoate dehalogenase from *Pseudomonas* sp. strain CBS3 can dehalogenate at least two different compounds carrying a chloro group and two nitro groups on the same aromatic ring. This reaction leads to the nitrophenols, which are more easily degradable by microorganisms. It is surprising that the activity with the nitro compounds was not higher than that with 4-chlorobenzoate, since in these compounds the nucleophilic exchange of the chlorine should be facilitated. The lower activity with the nitro compounds might be due to steric effects of the nitro groups.

#### ACKNOWLEDGMENTS

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