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# Purification and Characterization of Hydrolytic Haloalkane Dehalogenase from *Xanthobacter autotrophicus* GJ10

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**A new enzyme, haloalkane dehalogenase, was isolated from the 1,2-dichloroethane-utilizing bacterium *Xanthobacter autotrophicus* GJ10. The purified enzyme catalyzed the hydrolytic dehalogenation of *n*-halogenated $C_1$ to $C_4$ alkanes, including chlorinated, brominated, and iodinated compounds. The highest activity was found with 1,2-dichloroethane, 1,3-dichloropropane, and 1,2-dibromoethane. The enzyme followed Michaelis-Menten kinetics, and the $K_m$ for 1,2-dichloroethane was 1.1 mM. Maximum activity was found at pH 8.2 and 37°C. Thiol reagents such as *p*-chloromercuribenzoate and iodoacetamide rapidly inhibited the enzyme. The protein consists of a single polypeptide chain of a molecular weight of 36,000, and its amino acid composition and N-terminal sequence are given.**

*Xanthobacter autotrophicus* GJ10 is a nitrogen-fixing hydrogen bacterium (28) that is able to utilize 1,2-dichloroethane and some other halogenated alkanes for growth (8). Degradation of 1,2-dichloroethane proceeds via 2-chloroethanol, chloroacetaldehyde, chloroacetate, and glycolate. For this, the organism constitutively produces two different dehalogenases, one specific for halogenated alkanes and one for halogenated carboxylic acids. The former enzyme catalyzes the hydrolytic cleavage of 1,2-dichloroethane and a number of other short chain *n*-haloalkanes to produce 2-chloroethanol or the other corresponding alcohols and halide ions (7).

A number of enzymes that are able to dehalogenate haloalkanoic acids such as chloroacetic acid and 2-chloropropionic acid have been characterized (1, 4–6, 9, 10, 14, 17–20). A dichloromethane-dehalogenating glutathione S-transferase was recently purified from *Hyphomicrobium* DM2 (11). However, up to now, hydrolytic dehalogenation of a chlorinated alkane has only been described for the *X. autotrophicus* GJ10 enzyme (7). We now report the purification and a number of biochemical properties of this haloalkane-dehalogenating enzyme.

## MATERIALS AND METHODS

**Organism and growth conditions.** *X. autotrophicus* GJ10 (8) was grown aerobically at 30°C in a medium containing (per liter): 5.37 g of $Na_2HPO_4 \cdot 12H_2O$, 1.36 g of $KH_2PO_4$, 0.5 g of $(NH_4)_2SO_4$, 0.2 g of $MgSO_4 \cdot 7H_2O$, 5 ml of salts solution (8), and 1 ml of vitamins solution (8). The carbon source, citrate, was sterilized separately and added to a concentration of 1% (wt/vol). Cultures of 10 liters were grown in a Braun Biostat E fermentor (Melsungen, Federal Republic of Germany) at pH 7.0 and 70% oxygen saturation.

**Purification of haloalkane dehalogenase.** Cells were harvested by continuous centrifugation with a Sharples TIP and suspended in 10 mM Tris-sulfate buffer (pH 7.5) (Tris buffer). All further operations were carried out at 0 to 4°C, and all buffers contained 1 mM EDTA and 1 mM β-mercaptoethanol to protect the enzyme against inactivation.

**(i) Preparation of crude extract.** Washed cells (10 g [dry weight]) were resuspended in 1 volume of Tris buffer and disrupted by sonication (10 s/ml in an Ultrasonics W-375 sonicator at 300 W output). Unbroken cells and debris were removed by centrifugation for 30 min at 45,000 × *g*, and a crude extract was obtained.

**(ii) Ammonium sulfate fractionation.** The extract was diluted to 10 mg of protein per ml and fractionated by stepwise addition of solid ammonium sulfate to 40, 45, 50, 55, 60, and 80% saturation. After each step, the precipitate was collected by centrifugation for 10 min at 15,000 × *g*, dissolved in Tris buffer, and assayed for activity. The fraction containing the highest activity (60 to 80% precipitate) was dialyzed overnight against Tris buffer.

**(iii) DEAE-cellulose chromatography.** The dialyzed enzyme solution was applied to a DEAE-cellulose column (2 by 30 cm) equilibrated with Tris buffer. The column was washed with 20 ml of Tris buffer, and elution was carried out with a 0 to 1 M linear gradient of ammonium sulfate in Tris buffer (total volume, 600 ml; flow rate, 40 ml/h; fraction volume, 5.8 ml). The enzyme eluted at an ammonium sulfate concentration of 0.14 to 0.19 M. Active fractions were pooled and dialyzed overnight against 5 mM potassium phosphate buffer (pH 7.5).

**(iv) Hydroxylapatite chromatography.** The dialysate was applied to a hydroxylapatite column (2.3 by 6 cm) equilibrated with 5 mM potassium phosphate buffer, and the enzyme was eluted with a linear gradient of 5 to 100 mM potassium phosphate (pH 7.5) (total volume, 600 ml; flow rate, 45 ml/h; fraction volume, 5 ml). Active fractions eluted from 10 to 15 mM phosphate and were concentrated by adding solid ammonium sulfate to 80% saturation. The precipitate was collected by centrifugation for 10 min at 15,000 × *g* and dissolved in 3 ml of Tris buffer.

**(v) Gel filtration.** The enzyme was applied to a Sephadex G-100 column (2 by 80 cm) equilibrated with Tris buffer and then eluted with the same buffer (flow rate, 5 ml/h; fraction volume, 5 ml).

**Dehalogenase and protein assays.** Dehalogenase activities were routinely carried out with 1,2-dichloroethane as the substrate. A suitable amount of enzyme (about 60 mU for routine assays) in 25 μl of Tris buffer was incubated with 3.0 ml of 5 mM substrate in 50 mM Tris-sulfate buffer (pH 7.5) (substrate solution) at 30°C. Halide production (0 to 1 mM) was determined spectrophotometrically at 460 nm with mer-

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TABLE 1. Purification of haloalkane dehalogenase

| Step | Total protein (mg) | Total activity (U) | Sp act (U/mg of protein) | Yield (%) | Purification factor |
|---|---|---|---|---|---|
| Crude extract | 5,550 | 900 | 0.16 | 100 | |
| Ammonium sulfate | 466 | 447 | 0.96 | 50 | 6 |
| DEAE-cellulose | 59 | 279 | 4.7 | 31 | 29 |
| Hydroxylapatite | 30 | 166 | 5.5 | 18 | 34 |
| Sephadex G-100 | 19 | 114 | 6.0 | 12 | 37 |

curic thiocyanate and ferric ammonium sulfate as described by Bergmann and Sanik (2). One unit of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of halide per min.

**Molecular weight determination.** The molecular weight of denatured protein was determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis by the method of Laemmli (12). Bovine serum albumin (68,000), ovalbumin (45,000), aldolase (40,000), and chymotrypsinogen (25,000) were used as reference proteins. The gels contained 10% acrylamide and were stained with Coomassie brilliant blue G-250. Protein was determined by the method of Lowry et al. (15), with bovine serum albumin as the standard.

The molecular weight of the native enzyme was estimated by gel filtation on a Sephadex G-100 column as described under step (v) of the purification procedure. The column was calibrated with blue dextran, bovine serum albumin (68,000), ovalbumin (45,000), trypsin inhibitor (20,100), and cytochrome *c* (12,400).

**Localization of dehalogenase on polyacrylamide gels.** Dehalogenase was visualized after electrophoresis on 10% polyacrylamide slab gels as described by Weightman and Slater (27). All buffers contained 1 mM EDTA and 1 mM β-mercaptoethanol, and the gel was prerun overnight at 0°C. Samples of purified enzyme (40 μg of protein) were mixed with electrophoresis buffer (27) and loaded in preformed wells in the stacking gel. Electrophoresis was carried out at 30 mA and 4°C for 5 h. The gel was cut in three parts (A, B, and C). Part A was incubated in substrate solution for 30 min at 30°C, washed with distilled water, and placed in 0.1 M $AgNO_3$. Formation of AgCl precipitation bands indicated the regions of dehalogenase activity. Part B was stained with Coomassie brilliant blue. Part C was sliced into 12 equal pieces, and these were each incubated at 30°C in 3 ml of substrate solution. The presence of dehalogenase was determined by measuring the chloride concentration after 15 h of incubation.

**Isoelectric focusing.** The isoelectric point of the purified enzyme was estimated by isoelectric focusing by the method of O'Farrell (21) on 6.5% polyacrylamide gels containing 2% Ampholines in the pH range from 3.5 to 10. The pH profile of the gels was measured with a combined electrode for surface pH measurement (Ingold, Zurich) before the gels were stained with Coomassie brilliant blue.

**Amino acid analysis.** Purified enzyme was dialyzed against distilled water and subsequently hydrolyzed with 6 N HCl at 110°C under vacuum for 24, 48, and 72 h. The hydrolysates were analyzed for amino acid composition on a Kontron amino acid analyzer. Tryptophan was determined after 72 h of hydrolysis in the presence of mercaptoethanesulfonic acid (24).

**N-terminal amino acid sequence.** Automatic N-terminal sequence analysis was performed with a Beckman spinning cup sequencer model 890 c. Amino acids were identified by high-pressure liquid chromatography on Lichrosorb RP18 (5 μm; E. Merck AG, Darmstadt, Federal Republic of Germany).

**Materials.** DEAE-cellulose was from Whatman Ltd., Kent, England; Sephadex G-100 was from Pharmacia, Uppsala, Sweden; hydroxylapatite Bio-Gel HTP was from Bio-Rad Laboratories, Richmond, Calif.; Ampholines were obtained from LKB Produkter AB, Bromma, Sweden; and calibration proteins were from Sigma Chemical Co., St. Louis, Mo., or Boehringer GmbH, Mannheim, Federal Republic of Germany. Halogenated alkanes were obtained from Janssen Chimica Beerse, Belgium, and their purity was checked by gas chromatography. Methyl chloride and ethyl chloride were from Matheson Gas Products, Oevel, Belgium. Other chemicals were of analytical grade.

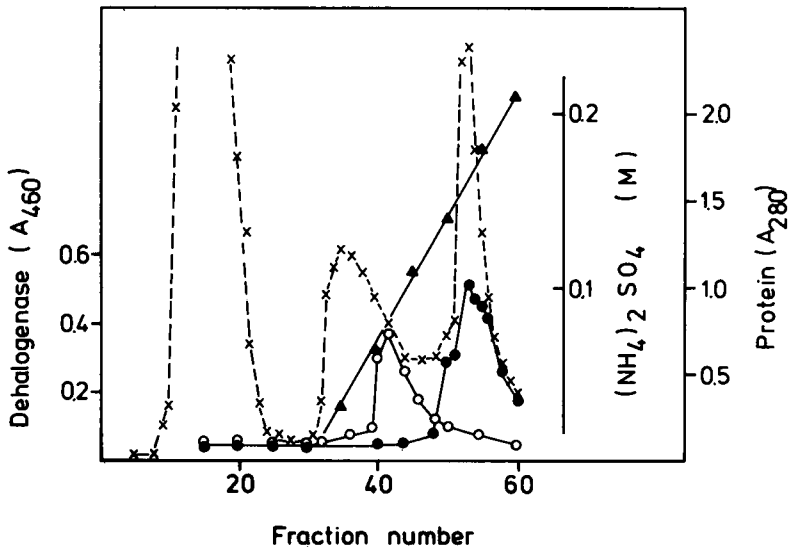


FIG. 1. Separation of haloalkane dehalogenase from haloalkanoate dehalogenase by DEAE-cellulose chromatrography. Fractions were assayed for protein at 280 nm (x), haloalkane dehalogenase (●), haloalkanoate dehalogenase (○), and $(NH_4)_2SO_4$ (▲) concentrations.

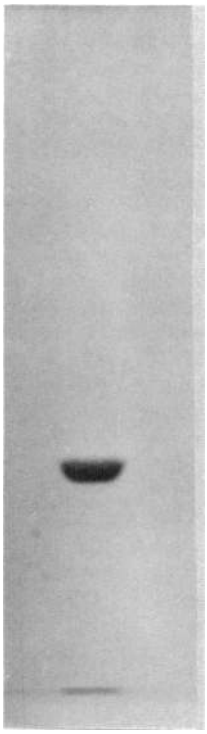

FIG. 2. SDS-polyacrylamide gel of purified dehalogenase. The stained band represents 10 μg of enzyme.

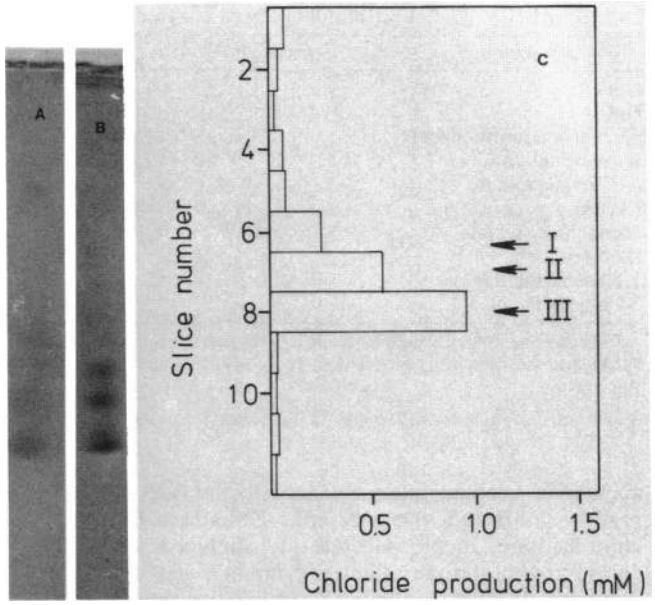


FIG. 3. Electrophoresis of dehalogenase under nondenaturing conditions. (A) Activity stain; (B) stained with Coomassie brilliant blue; (C) dehalogenase activity in gel slices.

## RESULTS

**Purification of haloalkane dehalogenase.** The purification scheme for haloalkane dehalogenase from *X. autotrophicus* GJ10 is summarized in Table 1. The enzyme was purified 37-fold, with an overall yield of 12%. This implies that the dehalogenase represents 2 to 3% of the total cellular protein. The elution profile of the DEAE-cellulose column (Fig. 1) shows that haloalkane dehalogenase is different from the haloalkanoic acid dehalogenase in the same organism, as was previously suggested on the basis of differences in heat lability (7).

After SDS-polyacrylamide gel electrophoresis, only one protein band was observed (Fig. 2). However, electrophoresis under nondenaturating conditions yielded three protein bands, and all three showed dehalogenase activity (Fig. 3). Apparently, the purified protein was homogeneous and represented dehalogenase but produced three bands as a result of conformational changes or a tendency to form multimers.

The purified enzyme was stored in 50 mM Tris-sulfate buffer (pH 7.5) containing 1 mM EDTA and 1 mM β-mercaptoethanol for several weeks at 4°C with less than 20% loss of activity.

**Molecular weight.** The molecular weight of the dehalogenase was estimated by gel filtration to be 31,000, and after electrophoresis on SDS-polyacrylamide gels, a value of 36,000 was obtained. This indicates that the enzyme consists of a single polypeptide chain with a molecular weight of 36,000.

**Substrate specificity.** The activity of haloalkane dehalogenase against various substrates was assayed by measuring the rates of halide release. The enzyme had a broad substrate specificity and hydrolyzed chlorinated, brominated, and iodinated *n*-alkanes (Table 2). The best substrate was 1,2-dichloroethane, followed by 1,2-dibromoethane and 1,3-dichloropropane. Haloalkanes with chains longer than four carbons and haloacetates were not hydrolyzed.

**Enzyme kinetics.** Maximum enzyme activity was obtained at pH 8.2 and a temperature of 37°C. From the temperature-activity curve, an activation energy of 55 kJ/mol could be calculated. The enzyme was rather heat labile and rapidly became inactive at temperatures of 50°C or above.

With 1,2-dichlorethane as the substrate, the enzyme followed Michaelis-Menten kinetics, and measurement of initial velocities with different concentrations revealed a $K_m$ of 1.1 mM and a $V_{max}$ of 10 μmol/min per mg of protein.

**Inhibitors.** The effects of substrate analogs and possible inhibitors on the activity of haloalkane dehalogenase are shown in Table 3. Thiol reagents such as $HgCl_2$, iodo-

TABLE 2. Substrate specificity of the enzyme

| Substrate | Relative activity (%)[a] |
|---|---|
| Chloromethane | 28 |
| Chloroethane | 24 |
| 1,2-Dichloroethane | 100 |
| 1-Chloropropane | 51 |
| 3-Chloropropene | 45 |
| 1,3-Dichloropropane | 80 |
| 1-Chlorobutane | 31 |
| Bromoethane | 24 |
| 1,2-Dibromoethane | 94 |
| 1-Bromopropane | 29 |
| 1-Iodopropane | 14 |

[a] The rates of halide liberation from the different substrates are expressed as percentages of the rate observed with 1,2-dichloroethane. No activity was found with dichloromethane, 1,1-dichloroethane, 1,1,2-trichloroethane, 2-chloropropane, 1,2-dichloropropane, 1-chloropentane, 1,6-dichlorohexane, 1,9-dichlorononane, 2-chloroethanol, chloroacetate, dichloroacetate, bromoacetate, dibromoacetate, 2-chloropropionate, or 3-chloropropionate.

TABLE 3. The effect of inhibitors on enzyme activity

| Reagent[a] | Concn (mM) | Inhibition (%) |
|---|---|---|
| None | | 0 |
| $HgCl_2$ | 1 | 100 |
| *p*-Chloromercuribenzoate | 0.001 | 100 |
| Iodoacetamide | 1 | 100 |
| *N*-Ethylmaleimide | 1 | 67 |
| EDTA | 1 | 0 |
| Monochloroacetate | 5 | 0 |
| Dichloroacetate | 5 | 0 |
| 1,1-Dichloroethane | 5 | 0 |
| 2-Chloroethanol | 5 | 0 |

[a] The enzyme was incubated for 5 min at 30°C with the compound indicated before determination of dehalogenase activity with 1,2-dichloroethane as the substrate.

acetamide, *p*-chloromercuribenzoate, and *N*-ethylmaleimide readily inhibited the enzyme. Substrate analogs like chloroacetate, dichloroacetate, 1,1-dichloroethane, and 2-chloroethanol did not affect enzyme activity.

**Isoelectric point, amino acid composition, and N terminus.** Isoelectric focusing of haloalkane dehalogenase yielded a pI value of 5.4 for the purified enzyme. Only one protein band was observed on the stained gel.

The amino acid composition of the enzyme is shown in Table 4. Predominant residues were glutamic acid, aspartic acid, alanine, and leucine, while proline content was also relatively high. Only one cysteine was found.

The following N-terminal amino acid sequence was obtained: Met-Ile-Asn-Ala-Ile-X-Tyr-Pro-Asp-Glx-. Methionine was absent in about 20% of the protein, as suggested by the premature identification of the succeeding residue during each degradation step. Since Edman degradation was somewhat inefficient without an unblocking procedure, methionine may be partly formylated, but we have not determined this directly. Automatic and manual Edman degradation yielded the same results for the first three residues.

## DISCUSSION

The haloalkane dehalogenase of *X. autotrophicus* GJ10 was purified to homogeneity. The enzyme appears to consist of a single polypeptide chain with a molecular weight of 36,000. This is of similar size to the haloacetate halidohydrolase from *Pseudomonas* sp. (33,000) (9) and somewhat larger than the haloacetate halidohydrolase from *Moraxella* sp. (26,000) (10) and the 2-haloacid dehalogenase from *Pseudomonas putida* (25,000) (17). However, the haloalkane dehalogenase described here is inactive towards halogenated carboxylic acids, and it is not clear whether the similarity in molecular size observed among some dehalogenases represents an evolutionary relationship. Up to five different dehalogenases have been described in soil bacteria (6). The *X. autotrophicus* GJ10 haloalkane dehalogenase is also clearly different from the dichloromethane-dehalogenating enzyme of *Hyphomicrobium* strain DM2 (11). The latter enzyme is a hexameric glutathione transferase that requires the presence of reduced glutathione for dehalogenase activity, and it is only active with dihalomethanes.

Dehalogenase activity was ascribed to the purified enzyme by determination of activity regions after polyacrylamide gel electrophoresis. The enzyme was separated in three protein bands under nondenaturing conditions, which possibly must be assigned to a tendency to form dimers or trimers. Gel filtration of the dehalogenase on Sephacryl S-200 occasionally yielded double peaks which both represented dehalogenase with equal mobilities on SDS-polyacrylamide gels. Furthermore, treatment of both peaks with chymotrypsin or *Staphylococcus aureus* V8 protease yielded identical digestion patterns upon electrophoresis (data not shown). Thus, splitting of dehalogenase activity in more bands during electrophoresis does not necessarily imply the presence of different polypeptides.

Several substrate analogs did not inhibit or inactivate the enzyme, but dehalogenase activity was strongly inhibited by thiol reagents. The same has been shown for some (1, 9, 10, 17) but not all (1, 5, 14, 18, 19) haloalkanoate dehalogenases. Thus, the only cysteine residue in the enzyme is probably involved in activity and located in the active center. A plausible mechanism for the dehalogenase reaction that includes a thiol group for activity was proposed by Goldman (4).

A number of halogenated alkanes were hydrolyzed by the enzyme, among which are the environmentally important chlorinated hydrocarbons methyl chloride, ethyl chloride, and 1,2-dichloroethane (16). These compounds are considered priority pollutants by the Environmental Protection Agency (23). Carbon-chlorine, carbon-bromine, and carbon-iodine bonds of *n*-monohaloalkanes are hydrolyzed by the enzyme, which does not require oxygen or cofactors for activity (7). The dehalogenase described here is in fact the first enzyme known to be capable of direct hydrolytic dehalogenation of chlorinated hydrocarbons. Other dehalogenation reactions include nucleophilic substitution of dichloromethane (3, 13, 25), which requires reduced glutathione, and oxidation (13, 22), which is dependent on reduced pyridine nucleotide. Furthermore, dehalogenation may occur at low redox potential under anaerobic conditions by reductive mechanisms (13, 26). Hydrolytic dehalogenation does not require energy and represents a simple route for detoxification since the organic character of halogen, which causes toxicity, is lost. Thus, organisms containing dehalogenases with activity towards chlorinated hydrocar-

TABLE 4. Amino acid composition of the enzyme

| Amino acid[a] | Content (mol%) | No. of residues/ enzyme molecule[b] |
|---|---|---|
| Aspartic acid | 10.7 | 34 |
| Threonine[c] | 6.0 | 19 |
| Serine[c] | 6.5 | 20 |
| Glutamic acid | 11.4 | 36 |
| Proline | 7.4 | 23 |
| Glycine | 6.8 | 21 |
| Alanine | 9.1 | 28 |
| Cysteine[d] | 0.3 | 1 |
| Valine | 3.9 | 12 |
| Methionine | 2.6 | 8 |
| Isoleucine | 3.5 | 11 |
| Leucine | 8.6 | 27 |
| Tyrosine | 3.0 | 9 |
| Phenylalanine | 7.7 | 24 |
| Lysine | 3.3 | 10 |
| Histidine | 2.0 | 6 |
| Arginine | 5.8 | 18 |
| Tryptophan | 1.3 | 4 |

[a] Values are averages for 24-, 48-, and 72-h hydrolysis.
[b] Values were calculated on the basis of a molecular weight of 36,000.
[c] Values were corrected to time zero of hydrolysis.
[d] Cysteine was determined as *S*-carboxymethyl cysteine.

bons could play an important role in the detoxification of environmental pollutants. However, no such enzymes with activity towards chlorinated aromatic hydrocarbons or chlorinated ethylenes have yet been described.

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