Purification and characterization of D-2-haloacid dehalogenase from *Pseudomonas putida* strain AJ1/23

JUDITH M. SMITH,* KAREN HARRISON and JOHN COLBY

School of Biology, Sunderland Polytechnic, Sunderland SR1 3SD, UK

(Received 29 August 1989; revised 12 December 1989; accepted 18 January 1990)

A D-2-haloacid dehalogenase was isolated and purified to homogeneity from *Pseudomonas putida* strain AJ1/23. The enzyme catalysed the stereospecific dehalogenation of the D-isomer of 2-chloropropionate. Using a new ion-chromatograph assay, the enzyme was found to catalyse the dehalogenation of short-chain 2-halocarboxylic acids. Maximum enzyme activity occurred at pH 9.5 and 50 °C and the enzyme was insensitive to most –SH reagents. The enzyme has an M_r of about 135000 and appears to be composed of four subunits of identical M_r .

Introduction

Many xenobiotic compounds have been developed by man for pest control. Due to their recalcitrant nature they persist in the biosphere and may have damaging effects. Many xenobiotic compounds are halogenated; over 60% of herbicides contain at least one chlorine atom and dehalogenases may play a crucial role in their biodegradation (Slater, 1982).

2-Haloacid dehalogenases (EC 3.8.1.2) have been isolated from several strains of *Pseudomonads* (Goldman *et al.*, 1968; Little & Williams, 1971; Motosugi *et al.*, 1982a, b; Slater *et al.*, 1979; Weightman *et al.*, 1982). They catalyse the reaction

where R is a short-chain alkyl group or hydrogen and X is a halogen other than fluorine.

Until recently, it was thought that there were three types of 2-haloacid dehalogenases: those enzymes which act only on the L-isomer of 2-monochloropropionate, those which act on both the D- and the L-isomers with inversion of configuration, and those which act on both isomers but with the configuration maintained (Weightman *et al.*, 1982). Recently, however, two 2-haloacid dehalogenases which act specifically on the D-isomer have been reported from separate sources. One enzyme was observed in *Pseudomonas putida* strain AJ1/23 (ICI

Patent no. 179603, Smith *et al.*, 1989); the other was isolated and partially purified from a strain of *Rhizobium* (Leigh *et al.*, 1988).

In this paper, we describe the purification to homogeneity of the enzyme from *P. putida* AJ1/23 and report some of its structural and kinetic characteristics.

Methods

Organism and growth. *Pseudomonas putida* AJ1/23, isolated from a soil sample taken from an area exposed to chloropropionate, was provided by Dr S. C. Taylor, ICI Biological Products Business, Billingham, Cleveland, UK. It was grown in a 50 litre Bioengineering fermenter using 35 litres of growth medium containing (g l⁻¹) K₂HPO₄ (1.2), KH₂PO₄ (0.624), (NH₄)₂SO₄ (1.0), K₂SO₄ (1.0), CaCl₂·2H₂O (0.05), MgSO₄·7H₂O (0.2), NaCl (0.1), and 1 ml of a trace elements solution containing (g l⁻¹) CuSO₄·5H₂O (0.18), MnSO₄·7H₂O (0.88), ZnSO₄·7H₂O (0.81), sterilized by autoclaving. After autoclaving, 10 ml l⁻¹ of a filter-sterilized, acidified solution of 3.5 g FeSO₄·7H₂O l⁻¹ was added. Then 8 ml l⁻¹ of a filter-sterilized solution of 2.5 M-racemic 2-chloropropionate (DL-2-CPA), which had been previously adjusted to pH 7.0 with NaOH, on ice, was added. Growth was estimated by measuring the culture optical density at 600 nm using a Pye-Unicam PU 8800 spectrophotometer. When the culture OD₆₀₀ was about 1.0, an additional 8 ml l⁻¹ 2.5 M-DL-2-CPA solution was added. At an OD₆₀₀ of about 2.5, the culture was harvested using a Alfa-Laval LAB 102B-05 solids-retaining separator. The cells were resuspended in 10 mM-Tris/H₃PO₄ buffer, pH 7.5 and centrifuged at 30000 g for 10 min at 4 °C in a MSE Hi-spin 21 centrifuge. The pelleted cells were stored at –70 °C in 25 g batches.

Preparation of crude cell extracts. Thawed cells were resuspended in 10 mM-Tris/H₃PO₄ buffer, pH 7.5 and sonicated with a MSE Uniprep 150 ultrasonic disintegrator using a 19 mm probe at amplitude 14 µm for ten 30 s bursts separated by 30 s cooling periods. The sonicate was centrifuged at 38720 g and 4 °C for 30 min to remove suspended material. The supernatant, referred to as crude extract, was stored at –70 °C or used immediately.

Abbreviation: 2-CPA, 2-chloropropionic acid.

Protein estimation. Protein in samples was determined by the method of Bradford (1976) with Bio-Rad protein assay reagent using BSA as a standard.

Purification. All operations except HPLC were done at 4 °C. All buffers contained 1 mM-DTT.

(i) **DEAE-Sephacel chromatography.** Crude extract was applied to a DEAE-Sephacel column (15 × 2.5 cm) equilibrated with 10 mM-Tris/H₃PO₄ buffer, pH 7.5. The column was washed with 30 ml of equilibration buffer and eluted at 0.5 ml min⁻¹ with a 0.01–0.3 M gradient of Tris/H₃PO₄ buffer, pH 7.5; 2 ml fractions were collected.

(ii) **Ammonium sulphate precipitation.** Active fractions were pooled and solid ammonium sulphate added to 50% saturation. The precipitate was collected by centrifugation at 14300 g for 10 min and resuspended in a small volume of 50 mM-Tris/H₃PO₄ buffer, pH 8.0. The preparation was then dialysed against this buffer for at least 6 h.

(iii) **Gel-filtration chromatography.** The dialysed enzyme was applied to an Ultrogel AcA-34 gel-filtration column (90 × 2.5 cm) equilibrated with 50 mM-Tris/H₃PO₄ buffer, pH 8.0 and eluted using this buffer at a flow rate of 0.3 ml min⁻¹. Fractions (5 ml) were collected; those containing dehalogenase were pooled and concentrated by precipitation with ammonium sulphate as above.

(iv) **HPLC gel filtration.** This was done at room temperature using a modular Gilson 302 HPLC system. Precipitated protein was resuspended in a small volume of buffer and dialysed overnight against 50 mM-sodium phosphate buffer, pH 7.2. Samples (200 µl) were applied to a TSK 3000SWG HPLC column, pre-equilibrated with sodium phosphate buffer and eluted at 0.5 ml min⁻¹. Fractions were collected at 1 min intervals.

Determination of purity. Samples of purified enzyme were run on 10% (w/v) polyacrylamide gels by the method of Laemmli (1970) and stained with methanolic Coomassie brilliant blue R250. Gels were dried onto filter paper using a Atto gel drier and scanned using a Beckman DU-70 spectrophotometer fitted with a film holder.

Chromatofocusing. This was done on PBE exchanger. DEAE-Sephacel eluate containing 35 mg protein was loaded onto a chromatofocusing column (1.5 × 27 cm) pre-equilibrated with 300 ml 0.02 M-imidazole/HCl buffer, pH 7.4, containing 1 mM-DTT. The column was eluted at a flow rate of 1 ml min⁻¹ with a pH 7.5 to pH 4.5 gradient of polybuffer 74; 2 ml fractions were collected. The fractions were measured for dehalogenase activity immediately after elution and their pH was measured on completion of the gradient.

Storage. Between purification stages, the enzyme was stored frozen at –70 °C or drop frozen in liquid nitrogen and then stored at –70 °C, both without significant loss of activity.

Measurement of dehalogenase activity. D-2-Haloacid dehalogenase activity was routinely determined by one of two methods.

(i) **L-Lactate dehalogenase linked spectrophotometric assay.** This assay was adapted from the lactate assay of Noll (1984). The principle of the assay is that the dehalogenase converts D-2-CPA to L-lactate; this in turn is oxidized to pyruvate by L-lactate dehydrogenase with concomitant reduction of NAD. The unfavourable equilibrium of this reaction is overcome by removal of pyruvate to form alanine by pyruvate:glutamate transaminase. Assay mixtures (0.5 ml) contained 350 µl distilled water, 15 µl 50 mM-NAD, 110 µl 0.5 M-glutamate/NaOH buffer, pH 10.0, 10 µl 1 M-DL-2-CPA, 50 U L-lactate dehydrogenase and 10 U pyruvate:glutamate transaminase. After prewarming to 30 °C, the reaction was started by the addition of enzyme and the formation of NADH measured at 340 nm using a Pye-Unicam PU 8800 spectrophotometer.

(ii) **Ion-chromatograph assay.** This method is based on the measurement of Cl⁻ in assay mixtures by HPLC. Ion chromatography was done using a Dionex QIC Analyser, fitted with a HPIC AS4A anion

exchange column. Further details of this analysis have been described by Smith *et al.* (1989).

M_r determination. The M_r of the native enzyme was determined by gel-filtration HPLC using a TSK G3000 SWG column. The column was calibrated using a Sigma MW-GF-200 kit, containing sweet potato β-amylase (M_r 200000), yeast alcohol dehydrogenase (150000), BSA (66000), carbonic anhydrase (29000) and cytochrome c (12400). Each standard (2 mg) was loaded and eluted individually. The void volume (V₀) of the column was 52.6 ml (elution volume of Dextran Blue 2000, M_r 2000000). The relative retention of each protein, measured as elution volume/void volume (V_e/V₀) was calculated and used to prepare a calibration curve.

Subunit M_r was determined by SDS-PAGE by the method of Laemmli (1970). Samples were run on a 8–20% (w/v) acrylamide gradient gel, calibrated using a Sigma MW-SDS-200 kit, containing the following proteins: myosin (M_r 205000), β-galactosidase (116000), phosphorylase b (97400), BSA (66000), egg albumin (45000) and carbonic anhydrase (29000). Samples and standards containing 1% (w/v) SDS, 1% (v/v) 2-mercaptoethanol and 20% (w/v) sucrose were boiled for 2 min. Bromophenol blue solution (5 µl) serving as a front marker was added to all samples and markers. The gel was stained with methanolic Coomassie brilliant blue R250.

Materials. DL-2-Chloropropionate was obtained from Fluka. Other halogenated compounds (Table 2) were bought from Aldrich. Rabbit L(+)-lactate dehydrogenase, *Leuconostoc mesenteroides* D(–)-lactate dehydrogenase, porcine pyruvic transaminase, and DTT were obtained from Sigma. DEAE-Sephacel, Ultrogel AcA-34, PBE exchanger and Polybuffer 74 were bought from Pharmacia.

Results

Purification of D-2-haloacid dehalogenase from P. putida AJ1/23

P. putida AJ1/23 was grown as a fed-batch culture in a mineral medium containing DL-2-CPA as sole carbon and energy source. A maximum concentration of 20 mM substrate was used, as higher concentrations were inhibitory to growth.

Of four different methods investigated in our laboratory for measuring dehalogenase activity, i.e. the Chloro-counter assay (Weightman *et al.*, 1982), colorimetric assay (Bergmann & Sanik, 1957), lactate dehydrogenase linked spectrophotometric assay (Noll, 1984) and ion-chromatograph assay (Smith *et al.*, 1989), the latter two methods were found to be the most useful. The lactate dehydrogenase linked assay was the quickest and was therefore useful in determining the distribution of dehalogenase in fractions collected after each purification step but could not be used for kinetic assays. The ion-chromatograph assay a sensitive assay developed in this laboratory was used to determine kinetic parameters of the enzyme and the specific activity of pooled samples (Table 1).

Table 1. Purification of D-2-haloacid dehalogenase from *P. putida* AJ1/23

D-2-Haloacid dehalogenase was purified from crude extract by the procedure described in Methods. Enzyme activity was determined using the ion-chromatograph assay. One unit of dehalogenase activity is defined as the amount of enzyme catalysing the release of $1 \mu\text{mol Cl}^- \text{min}^{-1}$.

Fraction	Activity (U ml ⁻¹)	Total activity (U)	Protein concn (mg ml ⁻¹)	Specific activity [U (mg protein) ⁻¹]	Purification (-fold)	Yield
Crude extract	6.0	420	12.0	0.5	1.0	100
DEAE-Sephacel eluate	7.5	360	1.3	5.8	11.6	85
Gel-filtration eluate	4.1	277	0.15	27.3	54.6	66
HPLC eluate	5.9	118	0.39	15.3	30.6	28

The dehalogenase was purified by the four steps described in Methods. As shown in Table 1, the enzyme was purified 50-fold by ion-exchange then gel-filtration chromatography. This preparation was 88% homogeneous as determined by gel-scanning of SDS-PAGE gels. Purification to 95% homogeneity was achieved by a further step using gel-filtration HPLC; however, this led to a significant decrease in specific activity. pH and temperature stability studies suggest these factors were not responsible.

On SDS-PAGE gradient gels, one clear band was observed giving an M_r value of 31800 ± 500 , calculated from mobility relative to standards. Holoenzyme M_r was determined by gel-filtration HPLC. Comparison of retention time with standards gave an M_r of 134490 suggesting that the enzyme is a tetramer.

Isoelectric point

The isoelectric point of the enzyme was determined by chromatofocusing. Dehalogenase eluted in a single peak around pH 5, but overnight storage under these conditions led to complete loss of activity. Attempts to recover active enzyme by immediate dialysis of the enzyme on elution against 10 mM-Tris/ H_3PO_4 buffer, pH 8.0, with or without prior concentration by ammonium sulphate precipitation, were unsuccessful.

Kinetic parameters of dehalogenase determined using variations of the ion-chromatograph assay

Effect of temperature. The highest measurable activity was recorded at 50 °C; at higher temperatures the enzyme was rapidly inactivated. This correlates well with the results of experiments into the stability of

enzyme at different temperatures (Fig. 1). Enzyme activity was very stable at 30 and 35 °C (later experiments showed no loss of activity at 30 °C and only 15% loss at 35 °C after 3 h incubation). However, above 40 °C loss of activity was readily observed, and at 56 °C rapid inactivation of the enzyme was accompanied by visible protein precipitation. Replotting the data from Fig. 1 yielded an Arrhenius activation energy for the reaction of 53 kJ mol⁻¹.

Effect of pH. The relationship between pH and enzyme activity is given in Fig. 2. Significant enzyme activity only occurred in alkali conditions which is consistent with the nature of the reaction mechanism. Maximum activity was observed at pH 9.5; outside the range pH 8.0–10.0, less than 50% of this activity was seen. Below pH 5, there was rapid loss of enzyme activity. The enzyme was stable over the broad range pH 6–9, being less so in strongly alkali conditions.

Substrate specificity. The range of compounds dehalogenated by the enzyme was determined by modification of the ion-chromatograph assay (Table 2). In a separate experiment, the concentration of methanol added to aid dissolution of non-polar compounds was found not to affect enzyme activity. In control experiments, each substrate was observed under experimental conditions omitting enzyme. No significant spontaneous hydrolysis was observed with any of the compounds tested. Where some did occur (in all cases <2% of substrate was hydrolysed over a 20 min incubation time) this rate was subtracted from observed enzyme reaction rates. The substrate range was fairly limited, there being a requirement for a free primary carboxyl group, the reactive halogen to be in the C-2 position and an overall chain length not exceeding four carbons. Activity with iodoacetate could not be determined by this method due to the nature of the iodide peak.

Fig. 1. Temperature stability of the enzyme. Enzyme samples (0.5 mg ml^{-1}) were placed in microfuge tubes and incubated individually at 35 ($\square$), 42 ($\bullet$) and 56 $^{\circ}\text{C}$ ($\circ$). Samples of assay mixtures were taken at intervals and enzyme activity determined using the standard ion-chromatograph assay.

Fig. 2. pH profile of the enzyme. The effect of pH on enzyme activity ($\square$) was determined in 200 mM buffers using the ion-chromatograph assay. For determination of pH stability ($\bullet$), concentrated enzyme ($2 \text{ mg protein, ml}^{-1}$) was incubated in 100 mM buffers for 20 min. After diluting 1/10 with 0.3 M-Tris/ H_3PO_4 buffer, pH 8.0, 100 mM-DL-2-CPA was added. The residual activity was then determined using the ion-chromatograph assay. The buffers used in both experiments were sodium acetate/acetate (pH 4 and 5), sodium phosphate (pH 6–8), Tris/ H_3PO_4 (pH 8.5–9.5) and glycine/NaOH (pH 10–11).

Table 2. Substrate specificity of the enzyme

Halogenated compounds (50 mM; 5 mM if no activity was first observed with 50 mM) were substituted for DL-2-CPA in standard assay mixtures. The rate of release of Cl^- or Br^- from the compounds was measured using the ion-chromatograph assay and compared to that with DL-2-CPA. All compounds tested having chiral centres were racemic mixtures. Those not soluble in water were first dissolved in the minimum amount of methanol. No activity was observed with 2,3-dichloropropionate, 3-chloropropionate, 2-chloropropionamide, chloroacetaldehyde, bromoethanol, 2-chloroethanesulphonate, 2-bromodecanoate, 2-chloropropene, epichlorohydrin, epibromohydrin, chloroacetonitrile and fluoroacetate. Activity with DL-2-CPA (100%) was $21.7 \text{ U (mg protein)}^{-1}$.

Substrate	Relative activity (%)
DL-2-Chloropropionate	100
Bromoacetate	405
DL-2-Bromopropionate	363.5
Chloroacetate	140
2-Bromobutyrate	13.2
2,2-Dichloropropionate	6.7
2-Chlorobutyrate	3.3

Fig. 3. Effect of pH on substrate K_m . The relationship between pH and K_m for DL-2-CPA for *P. putida* D-2-haloacid dehalogenase was determined using the following buffers (200 mM): sodium phosphate ($\square$); Tris/ H_3PO_4 ($\blacksquare$); glycine/NaOH ($\circ$). Apparent K_m values were calculated from Lineweaver-Burk double reciprocal plots.

Stereospecificity. Enzyme stereospecificity was determined by two different methods. Firstly, the extent of dehalogenation of a known amount of substrate was measured in well-buffered assay mixtures using the ion-chromatograph assay. Secondly, using the spectrophotometric assay, the activity of the enzyme with either D- or L-2-CPA was determined by linking with either rabbit L-lactate dehydrogenase or *Leuconostoc mesenteroides* D-lactate dehydrogenase. Following the reaction to completion, it was found that 46.7% of the substrate was dehalogenated. Activity was observed only with the combination of D-2-CPA and L-lactate dehydrogenase in assay mixtures. From this we assume that the enzyme is specific to the D-isomer and that the reaction results in inversion of configuration.

K_m for halo-acids. The relationship between pH and K_m for substrate was determined over the range pH 6.0–10.5. Dehalogenase activity within the range 1.0–10 mM-DL-2-CPA was determined using the ion-chromatograph assay. The results (Fig. 3) show that K_m broadly matches the pattern for specific activity of the enzyme with pH (Fig. 2). The K_m for D-2-CPA was assumed to be half that of DL-2-CPA, the substrate used in experiments.

Dehalogenase activity with certain other substrates was determined at pH 7.5 using the ion-chromatograph assay. The K_m for chloroacetate was 27.5 mM and for DL-2-bromopropionate was 1.99 mM.

Inhibitors. The effect of a variety of known enzyme inactivators on the activity of dehalogenase was determined using the ion-chromatograph assay (Table 3).

Discussion

2-Haloacid dehalogenases appear under various names in the literature: monobromoacetate dehalogenase (Tsang *et al.*, 1988), haloacetate halohydrinase (Kawa-

Table 3. *Effect of inhibitors on enzyme activity*

Enzyme activity was measured using the ion-chromatograph assay after prior incubation in standard assay mixtures in the presence of inhibitor, but the absence of substrate, for 10 min at 30 °C. Activity was measured after the addition of substrate and is given as a percentage of a control assay with no added inhibitor. 100% activity was 21.7 U (mg protein)⁻¹ with DL-2-CPA as substrate.

Inhibitor	Relative activity (%)
<i>N</i> -Ethylmaleimide (10 mM)	46
Fe ³⁺ (1 mM)	100
Fe ²⁺ (1 mM)	98
Cu ²⁺ (10 mM)	27
KCN (1 mM)	100
HgSO ₄ (1 mM)	100
<i>p</i> -Chloromercuribenzoate (0.1 mM)	111
HgCl ₂ (1 mM)	114
Dithionitrobenzoate (10 mM)	63
Iodoacetate (10 mM)	64
(1 mM)	100

saki *et al.*, 1981*a, b*), halidohydrolase (Little & Williams, 1971), DL-2-haloacid dehalogenase (Motosugi *et al.*, 1982*a*) and 2-halo acid dehalogenase (Motosugi *et al.*, 1982*b*). All have specificity for substrate of short carbon-chain length, a free primary carboxyl group and the reactive carbon in the C-2 position. However, their relative activities towards individual compounds are different. In this respect, D-2-haloacid dehalogenase from *P. putida* AJ1/23 most resembles the enzymes described by Motosugi *et al.* (1982*a*) and by Little & Williams (1971).

The *M_r* of *P. putida* AJ1/23 D-2-haloacid dehalogenase was determined to be 134000, composed of subunits of *M_r* 31800. This value is between 2- and 4-fold higher than that described for the above enzymes.

P. putida AJ1/23 D-2-haloacid dehalogenase displays a pH optimum of 9.5, identical to that of most 2-haloacid dehalogenases; it possesses a similar broad stability range, pH 6.0–10.0. The temperature optimum of 50 °C is also close to other quoted values.

Isoelectric points have been quoted only for haloacetate dehalogenases (Kawasaki *et al.*, 1981*a, b*) which, at 5.4 and 5.2 respectively, are very similar to the value 5.0 found for the *P. putida* AJ1/23 D-2-haloacid dehalogenase.

From published data, it appears that susceptibility to –SH reagents varies with the different groups of dehalogenases. (i) Those enzymes which dehalogenate fluoroacetate are very susceptible (Kawasaki *et al.*, 1981*a*). (ii) Enzymes which dehalogenate DL-2-CPA with inversion of configuration, are not susceptible, whilst (iii) those that retain configuration are sensitive (Motosugi *et al.*, 1982*a*; Weightman *et al.*, 1982). (iv) L-2-CPA dehalogenase susceptibility appears to depend on

the source of the enzyme (Little & Williams, 1971; Motosugi *et al.*, 1982*b*). D-2-Haloacid dehalogenase from *P. putida* AJ1/23 is resistant to inhibition by –SH reagents, and thus resembles the second group. DTT, which retains –SH groups in the reduced condition is required to maintain long-term enzyme stability. The enzyme may be partially purified in its absence, but with reduced activity. Activity can then be enhanced by adding DTT. Insensitivity of the enzyme to mercurials suggest that the –SH groups are not at the active site.

A mechanism for dehalogenation of 2-CPA resulting in inversion of configuration was proposed by Weightman *et al.* (1982). The active site configuration postulated has a positively charged pocket to bind the carboxylate group which is in close proximity to a basic group which will promote nucleophilic attack. This proposal is consistent with the substrate and pH range of *P. putida* AJ1/23 D-2-haloacid dehalogenase. The pH/activity profile of the enzyme (Fig. 2) suggests that binding of 2-CPA occurs even at high pH values. A fall in activity is seen above pH 10.0; thus the positively charged pocket binding the carboxylate group must be strongly basic. Lysine and arginine, with *pK_a* values of ~10 and >12.0 respectively (Metzler, 1977) may be present at this site. 2-CPA is a strong acid (*pK_a* 2.83; Weast, 1986) and thus is in the dissociated form throughout the pH range of enzyme activity. The enzyme shows a rapid increase in activity above pH 7.0; also, as indicated in Fig. 3, *K_m* for substrate over the pH range 6–10 is fairly constant. Below pH 7.0, *K_m* and activity both fall off rapidly. A histidine residue (*pK_a* 6.4–7.0; Metzler, 1977) may act as the basic group which promotes nucleophilic attack, as in the proposed mechanism.

In a series of experiments, Little & Williams (1971) found that 2,4,6-trinitrobenzene sulphonate inhibited halidohydrolase activity only marginally and both phenylmethanesulphonyl fluoride and *N*-acetylimidazole had no effect. They concluded that it was unlikely that free amino, seryl or tyrosyl residues were involved in the enzyme reaction. Susceptibility to photo-oxidation and the results of kinetic experiments were found to be consistent with the presence of an active histidine residue. Similar experiments could be done on the *P. putida* D-2-haloacid dehalogenase to help elucidate active-site structure.

We acknowledge financial assistance from ICI Biological Products Business, and Dr S. C. Taylor and Dr J. M. Liddell for their help and provision of *P. putida* strain AJ1/23. K. H. thanks SERC for a Quota Studentship.

References

- BERGMANN, J. G. & SANIK, J. (1957). Determination of trace amounts of chloride in naphtha. *Analytical Chemistry* **29**, 241–243.

- BRADFORD, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248-254.
- GOLDMAN, P., MILNE, G. W. A. & KEISTER, D. B. (1968). Carbon-halogen bond cleavage. III. Studies on bacterial halohydrases. *Journal of Biological Chemistry* **243**, 428-434.
- KAWASAKI, H., MIYOSHI, K. & TONOMURA, K. (1981a). Purification, crystallisation and properties of haloacetate halohydrase from *Pseudomonas* species. *Agricultural and Biological Chemistry* **45**, 543-544.
- KAWASAKI, H., TONE, N. & TONOMURA, K. (1981b). Purification and properties of haloacetate halohydrase specified by plasmid from *Moraxella* sp. strain B. *Agricultural and Biological Chemistry* **45**, 35-42.
- LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680-685.
- LEIGH, J. A., SKINNER, A. J. & COOPER, R. A. (1988). Partial purification, stereospecificity and stoichiometry of three dehalogenases from *Rhizobium* species. *FEMS Microbiology Letters* **49**, 353-356.
- LITTLE, M. & WILLIAMS, P. A. (1971). A bacterial halohydrase. Its purification, some properties and its modification by specific amino acid reagents. *European Journal of Biochemistry* **21**, 99-109.
- METZLER, D. E. (1977). *Biochemistry. The Chemical Reactions of Living Cells*, p. 59. New York: Academic Press.
- MOTOSUGI, K., ESAKI, N. & SODA, K. (1982a). Purification and properties of a new enzyme, DL-2-haloacid dehalogenase, from *Pseudomonas* sp. *Journal of Bacteriology* **150**, 522-527.
- MOTOSUGI, K., ESAKI, N. & SODA, K. (1982b). Purification and properties of 2-halo acid dehalogenase from *Pseudomonas putida*. *Agricultural and Biological Chemistry* **46**, 837-838.
- NOLL, F. (1984). In *Methods of Enzymatic Analysis*, vol. 6, *Metabolites*, 1, Carbohydrates, pp. 582-588. Edited by H. U. Bergmeyer. Weinheim, FRG: Verlag Chemie.
- SLATER, J. H. (1982). New microbes to tackle toxic compounds. *South African Journal of Science* **78**, 101-104.
- SLATER, J. H., LOVATT, D., WEIGHTMAN, A. J., SENIOR, E. & BULL, A. T. (1979). The growth of *Pseudomonas putida* on chlorinated aliphatic acids and its dehalogenase activity. *Journal of General Microbiology* **114**, 125-136.
- SMITH, J. M., HARRISON, K., COLBY, J. & TAYLOR, S. C. (1989). Determination of D-2-halopropionate dehalogenase activity from *Pseudomonas putida* strain AJ1/23 by ion chromatography. *FEMS Microbiology Letters* **57**, 71-74.
- TSANG, J. S. H., SALLIS, P. J., BULL, A. T. & HARDMAN, D. J. (1988). A monobromoacetate dehalogenase from *Pseudomonas cepacia* MBA4. *Archives of Microbiology* **150**, 441-446.
- WEAST, R. C. (editor) (1986). *CRC Handbook of Chemistry and Physics*, 67th edn. Boca Raton, Florida: CRC Press.
- WEIGHTMAN, A. J., SLATER, J. H. & BULL, A. T. (1979). The partial purification of two dehalogenases from *Pseudomonas putida* PP3. *FEMS Microbiology Letters* **6**, 231-234.
- WEIGHTMAN, A. J., WEIGHTMAN, A. L. & SLATER, J. H. (1982). Stereospecificity of 2-monochloropropionate dehalogenation by the two dehalogenases of *Pseudomonas putida* PP3: evidence for two different dehalogenation mechanisms. *Journal of General Microbiology* **128**, 1755-1762.