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Determination of haloalkane dehalogenase activity by capillary zone electrophoresis

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Abstract

A new sensitive method has been developed for the determination of haloalkane dehalogenase activity. The enzymatic reactions were carried out directly in thermostatted autosampler vials and the formation of product — bromide or chloride ions — was monitored by sequential capillary zone electrophoresis runs. The determinations were performed in a 75 μm fused-silica capillary using 5 m*M* chromate, 0.5 m*M* tetradecyltrimethylammonium bromide (pH 8.4) as a background electrolyte, separation voltage 15 kV (negative polarity) and indirect detection at sample wavelength 315 nm, reference wavelength 375 nm for brominated and chlorinated substrates, respectively 0.1 *M* β-alanine–HCl (pH 3.50) as a background electrolyte, separation voltage 18 kV (negative polarity) and direct detection at 200 nm for brominated substrates. The temperature of capillary was in both cases 25°C. The method is rapid, can be automated, and requires only small amount of enzyme preparation and substrate.

Keywords: Haloalkane dehalogenase; Enzymes; Haloalkanes

1. Introduction

Halogenated aliphatic hydrocarbons constitute one of the largest groups of environmental pollutants as a result of their widespread use as solvents, pesticides, herbicides, insecticides and chemical intermediates [1]. Because of their toxicity, bioconcentration and persistence, the ubiquitous distribution of halogenated compounds in the biosphere has caused public concern over the possible effects on the quality of life [2]. The study of the biochemistry of dehalogenation processes may help to understand and evaluate the potential for their degradation in nature [3]. Moreover, biotransformation of organic compounds with biocatalysts offer new routes for the synthesis of intermediates.

Haloalkane dehalogenases (EC 2.8.1.1) are group of enzymes involved in the biodegradation of these compounds by catalysing cleavage of the carbon–halogen bond [4]. A molecule of water is consumed per molecule of substrate and the reaction products are a primary alcohol, a halide and a proton:

$$CH_3(CH_2)_nX + H_2O \rightarrow CH_3(CH_2)_nOH + H^+ + X^-$$

Because of the significance of haloalkane dehalogenase for ecotoxicology and synthetic chemistry, many activity assays have been developed for this enzyme [5–12]. Most existing methods for determination of haloalkane dehalogenase enzymatic activity are based on measuring chloride production colorimetri-

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cally through chloride specific chemical reactions [5,6] or by ion-selective electrode [7]. However these methods have several drawbacks. The colorimetric methods employ toxic and corrosive reagents, the ion-selective electrodes equilibrate slowly and have the problem with interference by other ions.

In 1990–1991 capillary zone electrophoresis (CZE) was introduced as a method for sensitive separation and determination of inorganic anions [13–15]. Features like short separation time, high efficiency and sample throughput, minimal consumption of reagents and background electrolyte and minute sample requirement make CZE attractive for the determination of inorganic anions in a wide range of matrices.

In this work, a new sensitive method has been developed for the determination of haloalkane dehalogenase activity using CZE. The reactions were carried out directly in autosampler vials and sequential CZE runs monitored the formation of chloride or bromide ions.

2. Experimental

2.1. Materials and reagents

The 1,3-dibromopropane, 1-chlorobutane and tetradecyltrimethylammonium bromide (TTAB) were obtained from Aldrich (Milwaukee, WI, USA). All other chemicals and solvents were of analytical reagent grade, supplied from Sigma (St. Louis, MO, USA). All solutions were prepared with Milli-Q Academic water (Millipore, Milford, WA, USA) and filtrated through a 0.2 μm membrane filter.

2.2. Enzyme preparation

His-tagged hydrolytic haloalkane dehalogenase of *Sphingomonas paucimobilis* UT26 (LinB) purified to homogeneity was used as a model enzyme [12,16,17]. To overproduce LinB, the enzyme was expressed in *Escherichia coli* JM109 under isopropyl-β-thiogalactopyranoside (IPTG) induction. Transformed *E. coli* was cultured in 2 l of Luria broth at 37°C. IPTG was added the cells up a final concentration of 1 m*M* when the culture reached an optical density of 0.6 at 660 nm. The cells were harvested after 3 h incubation, resuspended in 50 m*M* phosphate buffer pH 7.5 containing 1 m*M* β-mercaptoethanol and 10% glycerol and disrupted by sonication. After centrifugation at 100 000 *g* for 1 h, the supernatant was applied on a Ni-NTA Sepharose column (QIAGEN, Hilden, Germany) equilibrated with 20 m*M* phosphate buffer (pH 7.5) containing 0.5 *M* sodium chloride and 10 m*M* imidazole. The unbound proteins were washed out by equilibrating buffer and the His-tagged LinB was eluted by the same buffer containing 0.5 *M* imidazole. The purified enzyme was stored in 50 m*M* phosphate buffer (pH 7.5) containing 1 m*M* β-mercaptoethanol and 10% glycerol at 0–4°C.

2.3. Capillary electrophoresis conditions

A Hewlett-Packard ^{3D}Capillary Electrophoresis system (Waldbronn, Germany) with a diode-array UV detector was used to carry out all CZE separations. The data were collected on a HP Vectra VL5 166 MHz personal computer using the Hewlett-Packard ^{3D}CE ChemStation software. A Polymicro Technology (Phoenix, AZ, USA) 64.5 cm (56.0 cm effective length) 75 μm fused-silica capillary was used for all separations. Injection was accomplished by an application of 50.0 mbar pressure for 4.0 s to the inlet vial in the case of long-end injection, or 50.0 mbar vacuum to the outlet vial for 4.0 s in the case of short-end injection. Temperature of the capillary was 25°C.

2.3.1. Method for chlorinated and brominated substrates [18]:

The determination was performed using 5 m*M* chromate, 0.5 m*M* TTAB (pH 8.4) as background electrolyte. No further adjustment of pH was needed. Separations were performed at 15 kV (negative polarity). Samples were detected at sample wavelength 315 nm with a bandwidth 10 nm, reference wavelength 375 nm with a bandwidth 20 nm. The capillary was washed with 0.1 *M* NaOH for 1 min, deionized water for 1 min and with background electrolyte for 2 min before each run.

2.3.2. Method for brominated substrates [19]:

The determination was performed using 0.1 *M* β-alanine–HCl (pH 3.50) as a background elec-

trolyte. Separations were performed at 18 kV (negative polarity). Samples were detected at 200 nm with a bandwidth 10 nm. The capillary was washed with background electrolyte for 1 min before each run.

2.4. Enzyme reaction monitoring

Enzyme reactions were carried out in 50 m*M* glycine buffer (pH 9.0). 0.5 ml of glycine buffer containing 10 m*M* 1,3-dibromopropane, 1-chlorobutane respectively, was placed in a polypropylene vial and incubated in the CZE system autosampler which was heated using a recirculating water bath. Only a short temperature incubation step for 2–3 min was performed because the glycine buffer was preincubated to the given temperature in water bath. The reaction was initiated by the addition of given amount of an enzyme. After enzyme addition, the autosampler vial was capped and the reaction was monitored by sequential CZE runs. The peak areas were measured using ChemStation software and the concentrations of halide were calculated from the calibration graphs. One unit of enzyme is defined as the amount that catalyses the production of one micromole of halide per min.

3. Results and discussion

Separation of inorganic anions by capillary electrophoresis in uncoated capillary can be performed at alkaline or acid pH. In the first case, a long-chain quaternary ammonium salt usually must be added as a flow modifier to the carrier electrolyte to reverse the direction of the electroosmotic flow [13–15,18,20–22]. By working at lower pH values, the ionization of capillary's silanol groups is suppressed and the electroosmotic flow is minimal [19,23]. No flow modifier is therefore needed. Both approaches were used to monitor the enzymatic activity of the haloalkane dehalogenase. The background electrolytes–5 m*M* chromate, 0.5 m*M* TTAB (pH 8.4) with indirect detection was used for brominated and chlorinated substrates, respectively 0.1 *M* β-alanine–HCl (pH 3.50) with direct detection for brominated substrates. In order to determine the enzyme activity with the highest precision; reproducibility, linearity and sensitivity of these electrolyte systems were evaluated by analyses of chloride and bromide standards prepared in 50 m*M* glycine buffer pH 9.0. To obtain rapid analysis, a short-end injection procedure was also tested [24]. Hydrodynamic injection from the short-end of the capillary is achieved by placing the sample vial at the outlet, the buffer vial at the inlet and then applying a vacuum. Electrophoresis is performed with reverse polarity to change the direction of migration. However the short-end injection cannot be used for chromate electrolyte, because the short 8.5 cm effective length was insufficient to resolve bromide and chloride ions from other inorganic anions, that could be present in the enzyme preparation (sulfate, phosphate, etc.). Fortunately these non-UV-adsorbing anions including chloride do not interfere with the direct detection of bromide using β-alanine electrolyte. The short-end injection in combination with this electrolyte resulted in reduction of the analysis time to about two min. That is useful especially for the samples containing

Table 1
Parameters of CZE method for chlorinated and brominated substrates using indirect detection[a]

Product	Cl^-	Br^-
Run time[b]	7 min	7 min
Migration time reproducibility ($n=10$)	0.64%	0.50%
Peak area reproducibility ($n=10$)	1.51%	3.03%
Linearity	50–1000 μ*M*	50–1000 μ*M*
Correlation coefficient	0.9997	0.9997
Limit of detection ($S/N \geq 3$)	0.8 μ*M*	0.7 μ*M*

[a] Samples: standards of sodium chloride or sodium bromide in 50 m*M* glycine buffer (pH 9.0). Separation conditions: fused-silica capillary [64.5 cm (56.0 cm effective length)×75 μm]; background electrolyte 5 m*M* chromate, 0.5 m*M* TTAB (pH 8.4); injection 50.0 mbar pressure for 4.0 s; separation voltage 15 kV (negative polarity); indirect detection at sample wavelength 315 nm with a bandwidth 10 nm, reference wavelength 375 nm with a bandwidth 20 nm; temperature of capillary 25°C.

[b] Including a 4 min flush time.

Table 2
Parameters of CZE method for brominated substrates using direct detection[a]

	Long-end injection	Short-end injection
Run time[b]	6 min	2 min
Migration time reproducibility ($n=10$)	0.51%	0.19%
Peak area reproducibility ($n=10$)	3.82%	2.69%
Linearity	10–1000 μ*M*	10–250 μ*M*
Correlation coefficient	0.9997	0.9986
Limit of detection ($S/N \geq 3$)	1.5 μ*M*	0.5 μ*M*

[a] Samples: standards of sodium bromide in 50 m*M* glycine buffer (pH 9.0). Separation conditions: fused-silica capillary [64.5 cm (56.0 cm effective length in the case of long-end injection, 8.5 cm in the case of short-end injection)×75 μm]; background electrolyte 0.1 *M* β-alanine–HCl (pH 3.50); injection 50.0 mbar pressure for 4.0 s to the inlet vial in the case of long-end injection, or 50.0 mbar vacuum to the outlet vial for 4.0 s in the case of short-end injection; separation voltage 18 kV (negative polarity); direct detection at 200 nm with a bandwidth 10 nm; temperature of capillary 25°C.

[b] Including a 1 min flush cycle.

higher haloalkane dehalogenase activity. Since the diffusion processes are reduced by minimising analysis time, sharper peaks were achieved and improved sensitivity consequently can be expected.

Tables 1 and 2 show that the precision of the CZE method using different electrolytes is good and the method is fully suitable to monitor halide released enzymatically by action of the haloalkane dehalogenase. As the overall performance of both electrolyte systems is comparable, their choice thus will depend on the purpose of activity determination.

Using the above described electrolyte systems and 1,3-dibromopropane as a substrate the measurement of haloalkane dehalogenase activity and its dependence on reaction time was studied. Overlaid electropherograms in Figs. 1–3 clearly show the process of formation of bromide upon the reaction time. As can be seen from the electropherograms of reaction mixture before and after addition of enzyme using indirect detection (Fig. 1), the enzyme extract contained low concentration of chloride (peak 2) and sulfate (peak 3). These ions come from previous

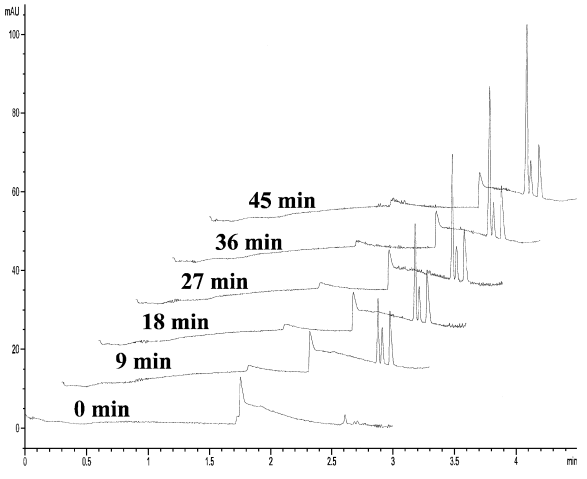

Fig. 1. CZE analysis of haloalkane dehalogenase catalysed reaction using indirect detection. Overlaid electropherograms show the reaction mixture of 10 m*M* 1,3-dibromopropane in 50 m*M* glycine buffer (pH 9.0) at 30°C before and 9, 18, 27, 36 and 45 min after addition of 10 μl of enzyme solution. The separation conditions are the same as in Table 1.

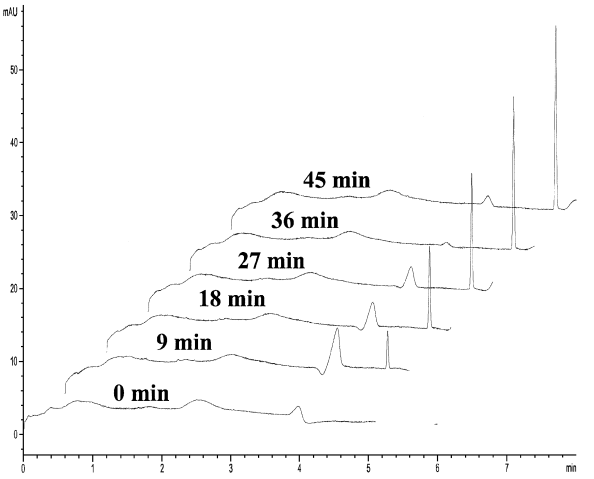

Fig. 2. CZE analysis of haloalkane dehalogenase catalysed reaction using long-end injection in combination with direct detection. The enzyme preparation was 4 times diluted with 50 m*M* phosphate buffer (pH 7.5). Separation conditions are the same as in Table 2. The other conditions are the same as in Fig. 1.

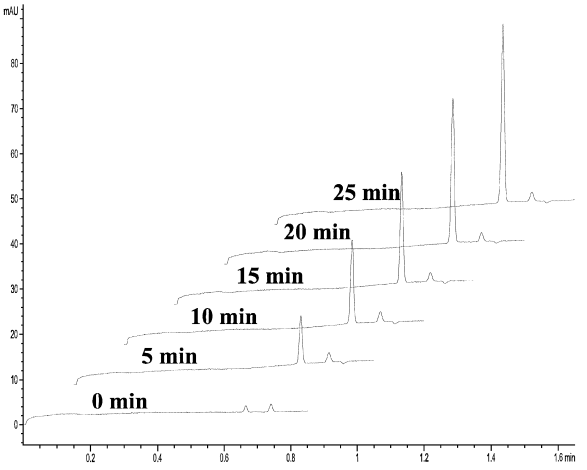

Fig. 3. CZE analysis of haloalkane dehalogenase catalysed reaction using short-end injection in combination with direct detection and. The conditions are the same as in Fig. 2.

purification steps where NaCl was used to suppress non-specific ionic interactions and sulfuric acid for the pH adjustment. Their higher concentration could interfere with this assay especially in the case of chlorinated substrates and dialysis of the sample would be necessary.

The plots of peak area of bromide versus the reaction time are shown in Fig. 4. As seen no loss of initial rate linearity occurred in approximately 40 min. Almost the same results were achieved using 1-chlorobutane as a substrate (data not shown). Analysis by CZE thus provided rapid monitoring of halide production enabling haloalkane dehalogenase activity to be determined.

A range of enzyme concentrations was tested as well. The reaction rates quantified by changes in peak area in time were calculated from data points collected within 10 min from the start of reaction. The reaction rates were found to be directly proportional to the enzyme concentration (Fig. 5), demonstrating that this assay technique is suitable for accurate estimation of the haloalkane dehalogenase activity in enzyme preparations. This was also confirmed by the comparison of the enzyme activities determined for the same substrate 1,3-dibromopropane by CZE method using different electrolyte systems. The values 0.129 IU ml^{-1} determined by conventional long-end injection, respectively 0.122 IU ml^{-1} by short-end injection, in combination with direct detection were in a good agreement with the value 0.131 IU ml^{-1} determined by indirect detection.

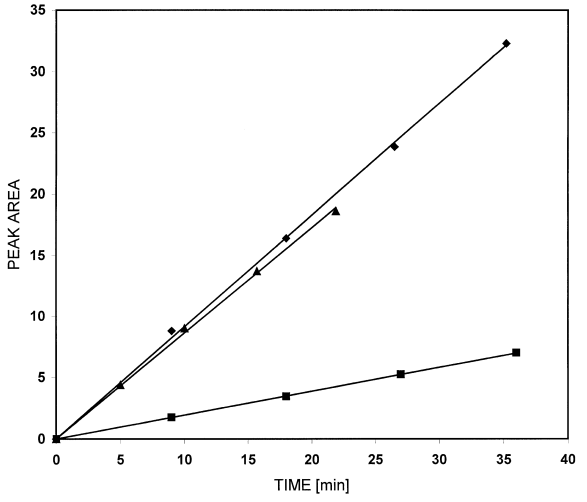

Fig. 4. Time dependent formation of bromide in haloalkane dehalogenase enzymatic reaction determined by CZE and indirect detection (■), direct detection in combination with a long-end (♦) or a short-end (▲) injection. The separation conditions are the same as in Tables 1 and 2.

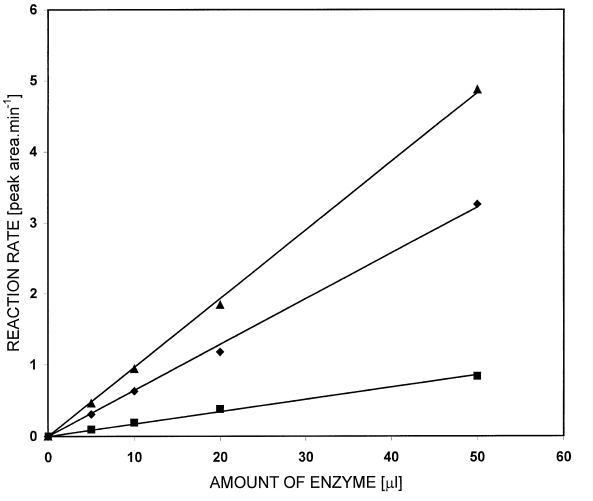

Fig. 5. Effect of enzyme concentration on the reaction rate (peak area min^{-1}) determined by CZE and indirect detection (■), direct detection in combination with a long-end (♦) or a short-end (▲) injection. 5, 10, 20 or 50 μl of enzyme solution (0.5 mg/ml) was added to the reaction mixture. The separation conditions are the same as in Tables 1 and 2.

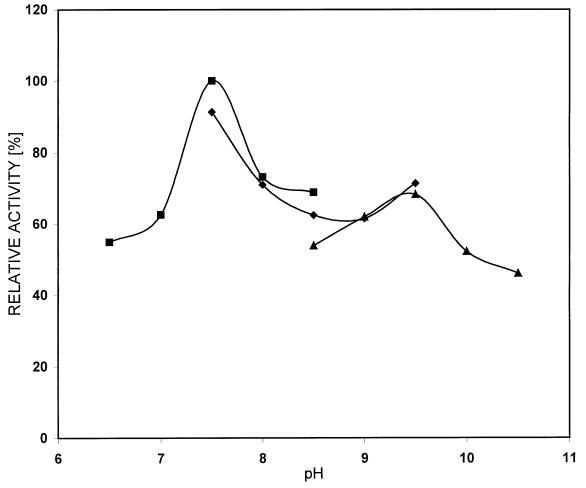

Fig. 6. pH dependence of haloalkane dehalogenase catalysed reaction. Reaction was performed using (■) 50 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 6.5–8.5); (♦) 50 m*M* Tris–HCl buffer (pH 7.5–9.5), or (▲) 50 m*M* glycine buffer (pH 8.5–10.5). Other conditions are the same as in Fig. 3.

The CZE method using short-end injection in combination with direct detection was applied to study some properties of the enzyme, such as the effects of pH and temperature on the enzymatic reaction with 1,3-dibromopropane as a substrate. The experimentally determined pH dependence of the haloalkane dehalogenase catalysed reaction is shown in Fig. 6. The influence of temperature on haloalkane dehalogenase activity was also studied (Fig. 7). An optimum was observed around 45°C beyond which rapid enzymatic denaturation occurred.

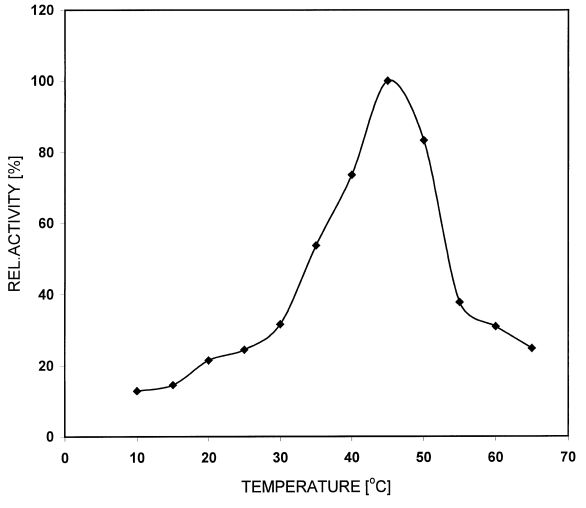

Fig. 7. Influence of temperature on haloalkane dehalogenase catalysed reaction. Reactions were performed at given temperature as described in Fig. 3.

4. Conclusions

A simple and rapid method for monitoring of the haloalkane dehalogenase activity has been developed. Compared to spectrophotometric and other assays, the capillary electrophoretic method is rapid, can be automated, and requires only small amount of enzyme preparation and substrates. Moreover, the enzymatic reaction can be monitored with high sensitivity and reproducibility. While the method was developed for determination of activity of haloalkane dehalogenase acting on haloaliphatic compounds, it could be used to monitor the activities of other dehalogenating enzymes.

Acknowledgements

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