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Determination of rhodanese enzyme activity by capillary zone electrophoresis

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Abstract

A new sensitive method has been developed for the determination of rhodanese activity. The enzymatic reactions were carried out directly in thermostatted autosampler vials and the formation of SCN^- was monitored by sequential capillary zone electrophoretic runs. The determinations were performed in a 75-μm fused-silica capillary using 0.1 *M* β-alanine–HCl (pH 3.50) as a background electrolyte, a separation voltage of 18 kV (negative polarity), a capillary temperature of 25°C and direct detection at 200 nm. Short-end injection or long-end injection procedures were used for sample application. The method is rapid, able to be automated and requires only small amounts of sample and substrates, which is especially important in the case of highly toxic cyanide. The developed capillary electrophoretic method also has great potential for thiocyanate determinations in other applications.

Keywords: Injection methods; Buffer composition; Rhodanese; Enzymes; Sulfurtransferases; Thiosulfate; Thiocyanate

1. Introduction

The major route of biological cyanide detoxification is by conversion to thiocyanate, which is relatively non-toxic [1]. This conversion obviously requires a source of sulfane sulfur, i.e. divalent sulfur bound only to other sulfur, since this is the form that can react with cyanide to produce thiocyanate. Such sulfane sulfur atoms occur in various compounds in biological systems [2]. These include inorganic thiosulfate anion and both of its oxidation products, the polythionates and their organic analogs, the thiosulfonates. In addition, the persulfides and polysulfides belong to this group and so does the staggered eight-membered ring of sulfur atoms. All of these sulfane-containing materials undergo rapid equilibration in vivo.

A number of distinct enzymes, the sulfurtransferases, have been shown to catalyse reactions that either use or produce sulfane sulfur [3]. These enzymes should be considered as possible active participants in cyanide detoxification as well as in other processes that may compete with cyanide detoxification for sulfane sulfur.

The enzyme rhodanese (thiosulfate:cyanide sulfurtransferase, EC 2.8.1.1), so named by Lang [4], is the longest-known, most studied and best-understood sulfurtransferase [5–10]. It catalyses transfer of the sulfane sulfur of thiosulfate to an acceptor, which is normally cyanide in the standard assay, and is likely to be cyanide under some physiological conditions:

$$S_2O_3^{2-} + CN^- \rightarrow SCN^- + SO_3^{2-}$$

Rhodaneses are practically ubiquitous enzymes; their

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activity has been detected in several species ranging from microorganisms through fungi, plants, and animals to man.

Because of the significance of rhodanese to fundamental and applied toxicology, many assay methods have been developed for rhodanese. Assay methods include spectrophotometric [11–13] and spectrofluorimetric [14] assays and measuring cyanide consumption or thiocyanate production using ion-selective electrodes [15,16]. All of these systems have associated limitations.

In this work, a new sensitive method has been developed for the determination of rhodanese activity. The reaction was carried out directly in a thermostatted autosampler vial and sequential capillary zone electrophoretic (CZE) runs monitored the formation of thiocyanate. This is the first paper describing the application of CZE for the determination of the activity of the enzyme utilising inorganic substrates.

2. Experimental

2.1. Materials and reagents

The enzyme rhodanese from bovine liver was obtained from Sigma (St. Louis, MO, USA). All other chemicals and solvents were of analytical reagent grade, supplied by Fluka (Buchs, Switzerland). All solutions were prepared with Milli-Q Academic water (Millipore, Milford, MA, USA) and was filtered through a 0.2-μm membrane filter.

2.2. Capillary electrophoresis conditions

A Hewlett-Packard ^{3D}Capillary Electrophoresis system (Hewlett-Packard, Germany) with a diode array UV detector was used to carry out all CZE separations. Data were collected on a HP Vectra VL5 166 MHz personal computer using the Hewlett-Packard ^{3D}CE Chemstation software. A Polymicro Technologies (Phoenix, AZ, USA) fused-silica capillary [64.5 cm (56.0 cm effective length)×363 μm O.D.× 75 μm I.D.] was used for all separations. Injection was accomplished by the application of 50.0 mbar of pressure to the inlet vial, or a 50.0-mbar vacuum to the outlet vial for 4.0 s. Samples were detected using a diode array detector at 200 nm with a bandwidth of 20 nm. The capillary was washed with the background electrolyte for 3 min after each run, although this was reduced to 1 min for the study of faster enzyme reactions.

2.3. Enzyme reaction monitoring

The enzymatic reactions were carried out in 50 m*M* 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) buffer (pH 8.5). 2-Morpholinoethanesulfonic acid (MES) buffer (50 m*M*) and 50 m*M* borate buffer were used for the study of the effect of pH on the enzymatic reaction.

Lyophilized rhodanese was dissolved in 50 m*M* HEPES buffer (pH 8.5) and stored at 4°C prior to use. One unit of enzyme is defined as the amount that catalyses the production of one micromole of thiocyanate per min.

2.3.1. Capillary electrophoretic measurements

A 0.5-ml volume of 50 m*M* HEPES buffer (pH 8.5) containing 2.5 m*M* sodium thiosulfate and a given amount of enzyme solution was placed in a polypropylene vial and incubated in the CZE system's autosampler, which was heated using a recirculating water bath. Only a short temperature incubation step of 2–3 min was performed because the HEPES buffer was preincubated to the given temperature in a water bath. The reaction was initiated by the addition of 25 μl of 0.5 *M* potassium cyanide. After the addition of potassium cyanide, 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 thiocyanate were calculated from the calibration graphs.

2.3.2. Continuous spectrophotometric measurements [12]

A final reaction mixture in a 1-cm cuvette contained 1.5 ml of HEPES buffer (pH 8.5), 0.5 ml of 0.3 *M* sodium thiosulfate, 50 μl of 4 m*M* 2,6-dichlorophenolindophenol (DCPIP), 0.1 ml of 16 m*M* methylphenazonium methyl sulfate (P*M*S), enzyme solution and water to a total volume of 2.95 ml. After incubation for 2–3 min, the reaction was initiated by the rapid addition and mixing of 50 μl of

2.0 *M* potassium cyanide. The assay was run in a thermostatted cell holder of a UV 3000 spectrophotometer (Shimadzu, Japan). The reduction of DCPIP was followed at 600 nm using a millimolar extinction coefficient of 20.6 [17].

3. Results and discussion

3.1. Optimization of CZE analysis

Inorganic anions are almost always determined by capillary electrophoresis at an alkaline pH [18–20]. However, 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. By working at lower pH values, the capillary's silanol groups are hardly ionized and the electroosmotic flow is minimal [21]. No flow modifier is therefore needed. Moreover, the problem with a high concentration of cyanide in the sample matrix is eliminated because cyanide is at an acid pH in molecular form, i.e. HCN ($pK_{a\,HCN} = 8.68$) [22], the electrophoretic mobility of which is zero.

In order to monitor the enzymatic activity of rhodanese, the optimal conditions for capillary electrophoresis of thiosulfate and thiocyanate were determined. A standard solution of thiosulfate and thiocyanate was prepared in 50 m*M* HEPES buffer (pH 8.5) containing 25 m*M* KCN. The background

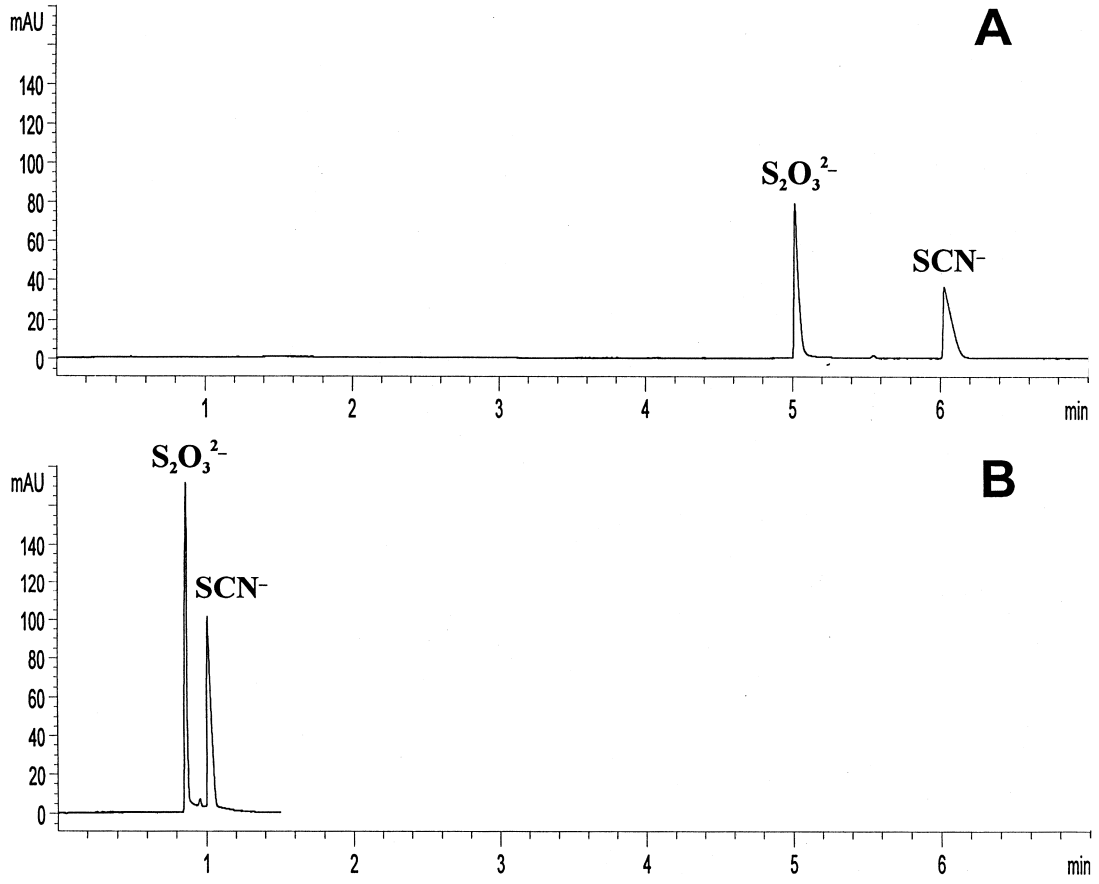

Fig. 1. CZE separations of rhodanese substrates and product [2.5 m*M* sodium thiosulfate and 1 m*M* sodium thiocyanate in 50 m*M* HEPES buffer (pH 8.5) containing 25 m*M* potassium cyanide]. Separation conditions: 0.1 *M* β-alanine–HCl (pH 3.50); separation voltage, 18 kV (negative polarity); fused-silica capillary [64.5 cm (56.0 cm effective length)×75 μm I.D.], direct detection at 200 nm, temperature of capillary, 25°C. (A) Conventional long-end injection for 4 s at 50 mbar. (B) Short-end injection for 4 s at −50 mbar.

Table 1
Comparison between short-end and conventional long-end injection procedures

	Short-end injection	Long-end injection
Run time[a]	3 min	8 min
Migration time reproducibility ($n=10$)	0.11%	0.04%
Peak area reproducibility ($n=10$)	0.33%	0.82%
Linearity	50–5000 μ*M*	50–5000 μ*M*
Correlation coefficient	0.9993	0.9998
Limit of detection	2.5 μ*M*	5 μ*M*

[a]Including a 1-min flush cycle.
Samples: standards of sodium thiocyanate in 50 m*M* HEPES buffer (pH 8.5) containing 2.5 m*M* sodium thiosulfate and 25 m*M* potassium cyanide.
Separation conditions: 0.1 *M* β-alanine–HCl (pH 3.50), a separation voltage of 18 kV (negative polarity), a fused-silica capillary [64.5 cm (56.0 cm effective length)×75 μm I.D.], direct detection at 200 nm, and a capillary temperature of 25°C.

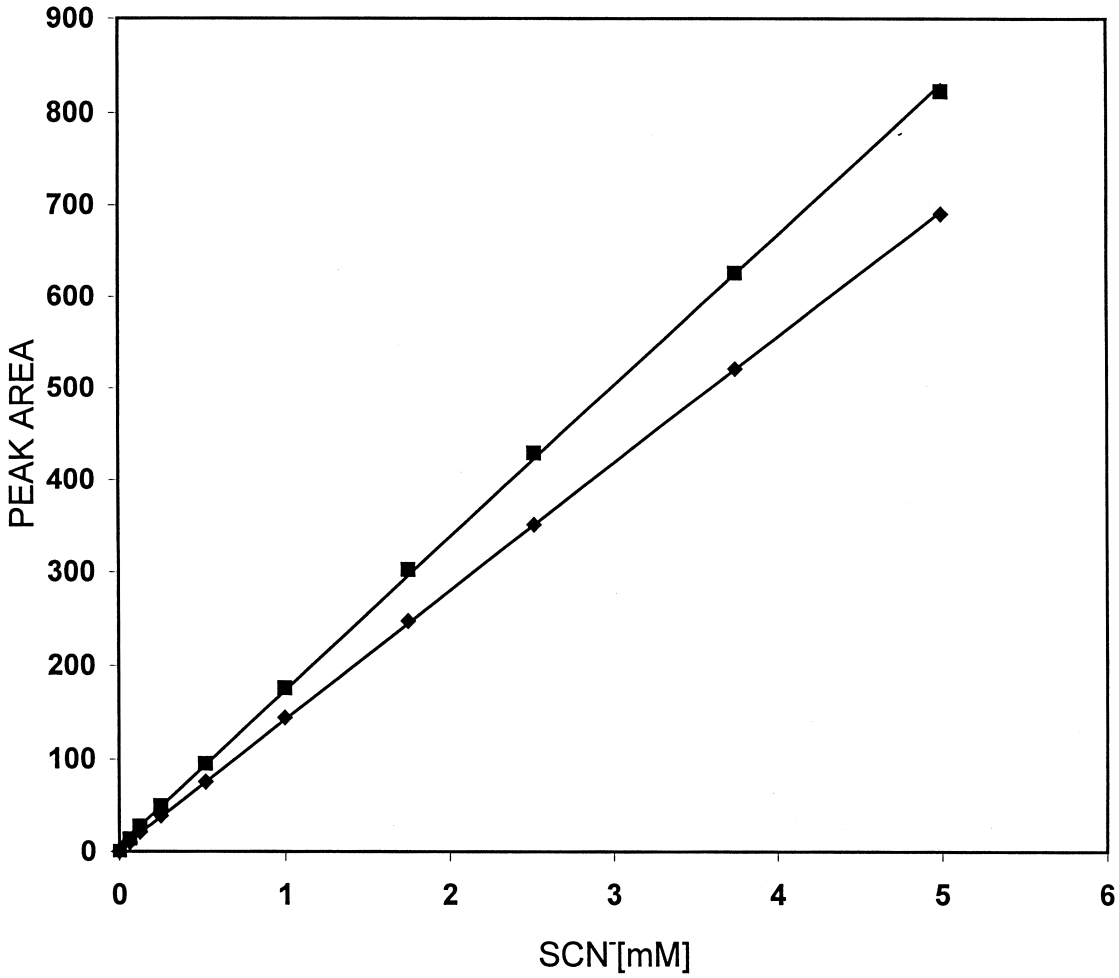

Fig. 2. Standard calibration curves for quantification of thiocyanate using the short-end injection procedure (■) and the long-end injection procedure (♦).

electrolytes, prepared by adding hydrochloric acid to β-alanine, were used at pH values ranging from about pH 2.0 to 4.0. We investigated the effects of the pH and ion strength of the background electrolyte, the separation voltage and the temperature of the capillary on separation and efficiency. Upon changing those conditions, the resolution and the number of theoretical plates of thiosulfate and thiocyanate were calculated, as described elsewhere [23]. Peak identification was accomplished by comparing the electrophoretic mobilities of suspected peaks with those of authentic standards.

Fig. 1A shows an electropherogram of a standard solution under optimal conditions of 0.1 *M* β-alanine–HCl (pH 3.5), at a separation voltage of 18 kV (negative polarity) and a capillary temperature of 25°C. As shown in Fig. 1A, more than adequate resolution of the two anions was obtained in 7 min.

To obtain rapid analysis, a short-end injection procedure was also tested [24]. Hydrodynamic injection from the short-end of the capillary was achieved by placing the sample vial at the outlet, the buffer vial at the inlet and then applying a vacuum. Electrophoresis was performed with reverse polarity to change the migration direction. The identical hydrodynamic injection and electrophoresis conditions using a short-end injection are shown in Fig. 1B. The short (8.5 cm) effective length was sufficient to resolve both anions, and the analysis time was reduced to about 2 min. This is especially useful for samples containing higher rhodanese activity. As diffusion processes are reduced by minimizing the analysis time, sharper peaks were achieved and improved sensitivity can be expected.

3.2. Method validation

The reproducibility, linearity and sensitivity of the method were tested (Table 1). Standards of thiocyanate prepared in 50 m*M* HEPES buffer (pH 8.5) containing 2.5 m*M* sodium thiosulfate and 25 m*M* potassium cyanide were analysed using conventional long-end injection as well as short-end injection procedures. Table 1 shows the excellent reproduci-

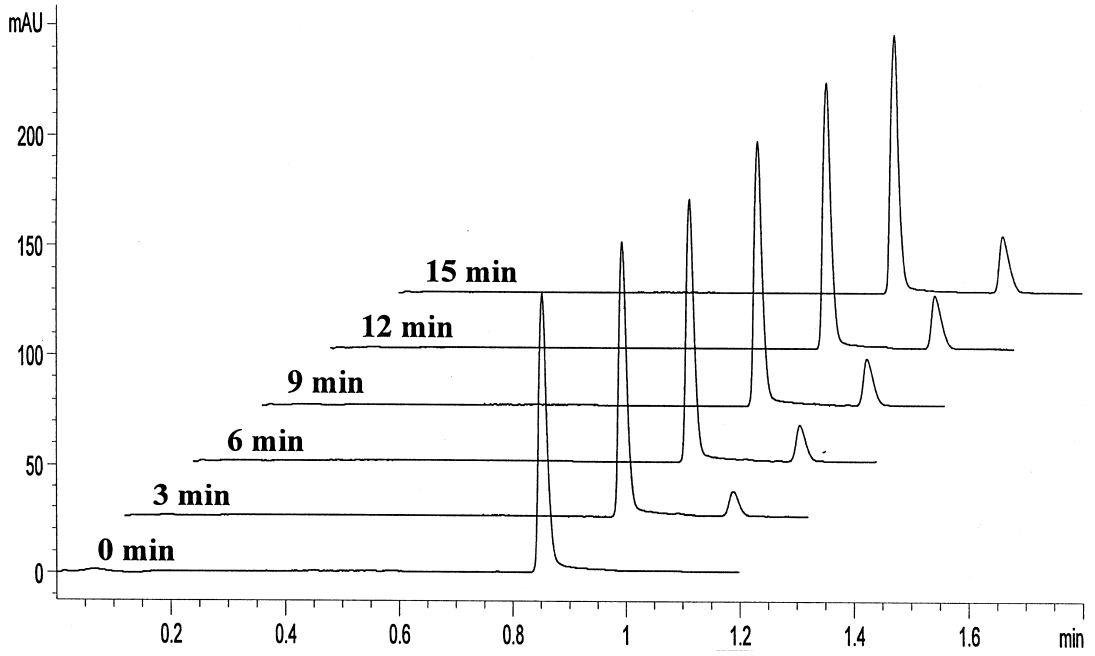

Fig. 3. CZE analysis of rhodanese-catalysed reactions. Overlaid electropherograms show the enzymatic reaction under standard conditions of 2.5 m*M* sodium thiosulfate and 25 m*M* potassium cyanide in 50 m*M* HEPES buffer (pH 8.5) and 30°C at 3, 6, 9, 12 and 15 min after the addition of potassium cyanide. A 2.5-μl volume of enzyme solution (0.8 mg/ml) was added to the reaction mixture. CZE conditions as in Fig. 1B.

bility obtained for migration time and peak area, which is probably the result of the suppressed electroosmotic flow. The calibration graphs (Fig. 2) were linear over the range 50 μ*M*–5 m*M* thiocyanate, with correlation coefficients better then 0.999. The detection limits were in the range 2.5–5 μ*M* at a signal-to-noise ratio of three.

The developed method has also great potential for thiocyanate determination in other applications because of better quantitative parameters in comparison

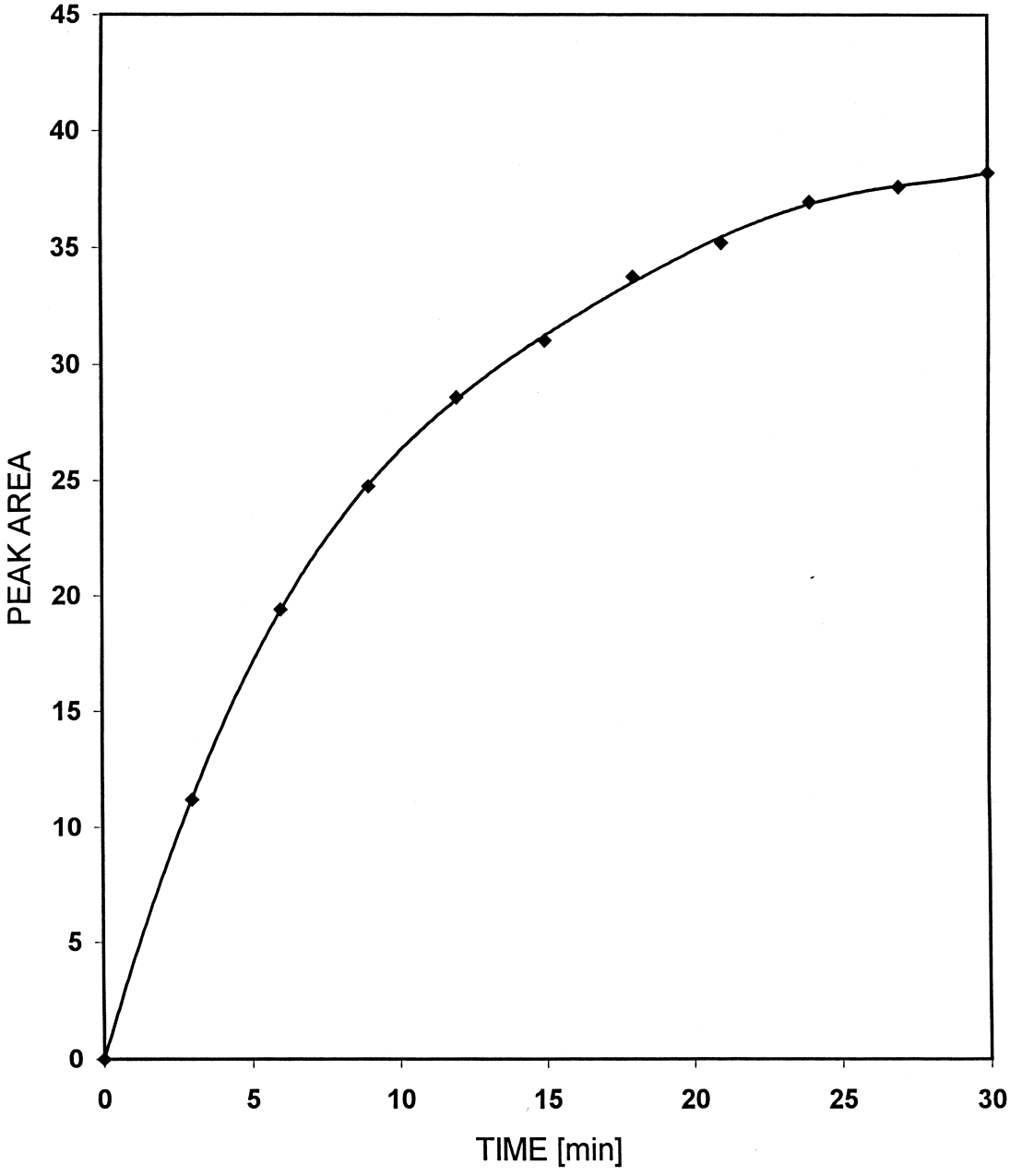

Fig. 4. Time-dependent formation of thiocyanate in rhodanese's enzymatic reaction. Conditions as in Fig. 3.

with CZE and micellar electrokinetic chromatography (MEKC) methods [25–28] published so far.

3.3. Measurement of activity and properties of rhodanese using CZE

Using the optimal conditions obtained from the above investigations, we have studied the measurement of rhodanese activity and its dependence on reaction time. A short-end injection procedure was used in all separations.

The overlaid electropherograms in Fig. 3 clearly show the process of formation of thiocyanate upon the reaction time. Analysis by CZE thus provided rapid separation of both the substrate, thiosulfate, and the product, thiocyanate, enabling rhodanese activity to be determined. Fig. 4 shows a plot of peak area of thiocyanate versus the reaction time. As can be seen, a loss of initial rate linearity occurred in approximately 10 min. A similar phenomenon was observed using other methods for the assay of rhodanese activity [12,15]. This is probably caused by inactivation of the enzyme, since changes in the concentration of substrates are negligible.

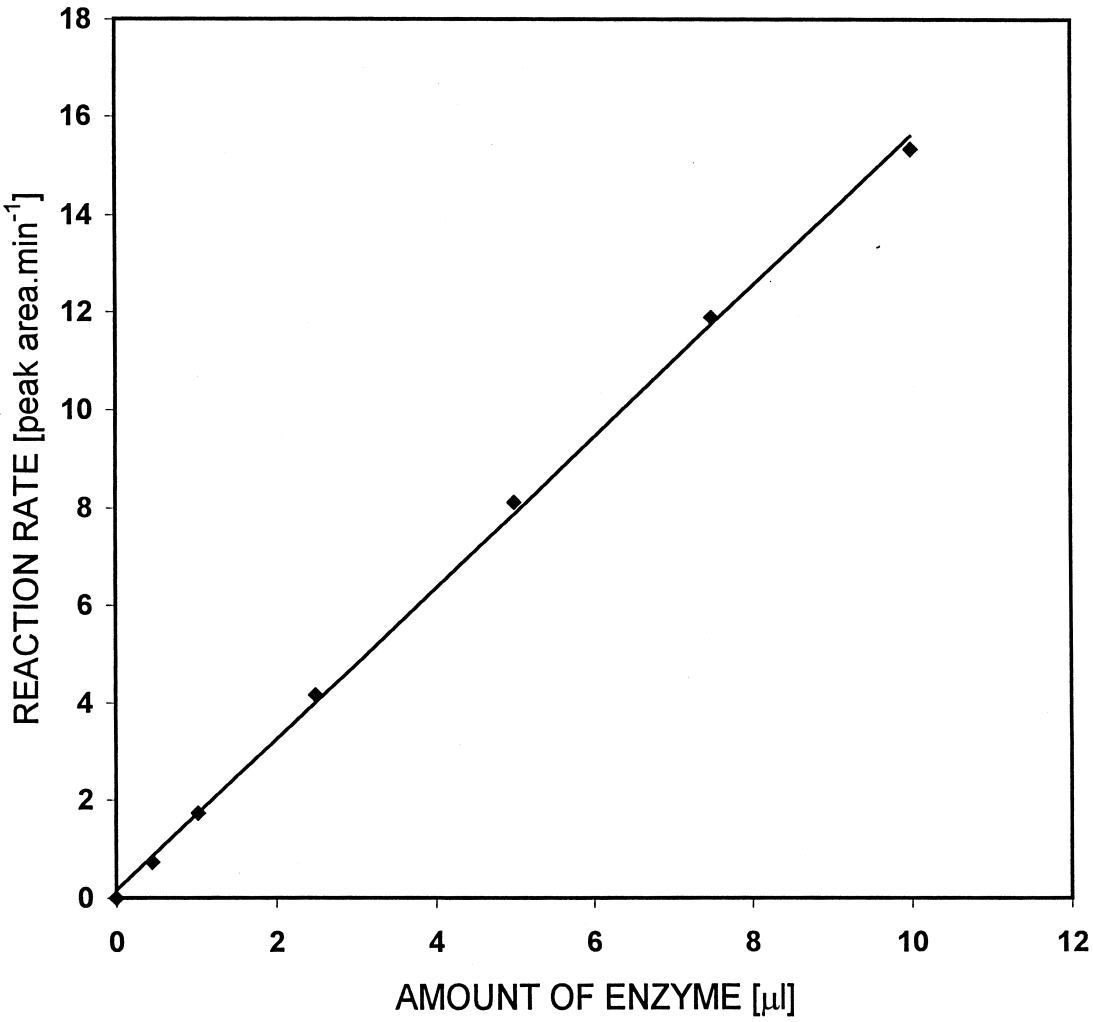

Fig. 5. Effect of enzyme concentration on the reaction rate (peak area/min). Enzyme solution (0.5, 1.0, 2.5, 5 or 10 μl; 0.8 mg/ml) was added to the reaction mixture. Other conditions as in Fig. 3.

A range of enzyme concentrations was tested as well. Reaction rates (peak area/min) were calculated from data points collected within 6 min from the start of the reaction. The reaction rate was found to be directly proportional to the enzyme concentration (Fig. 5), demonstrating that this assay technique is suitable for accurately estimating rhodanese activity in enzyme extracts. We also checked the enzyme

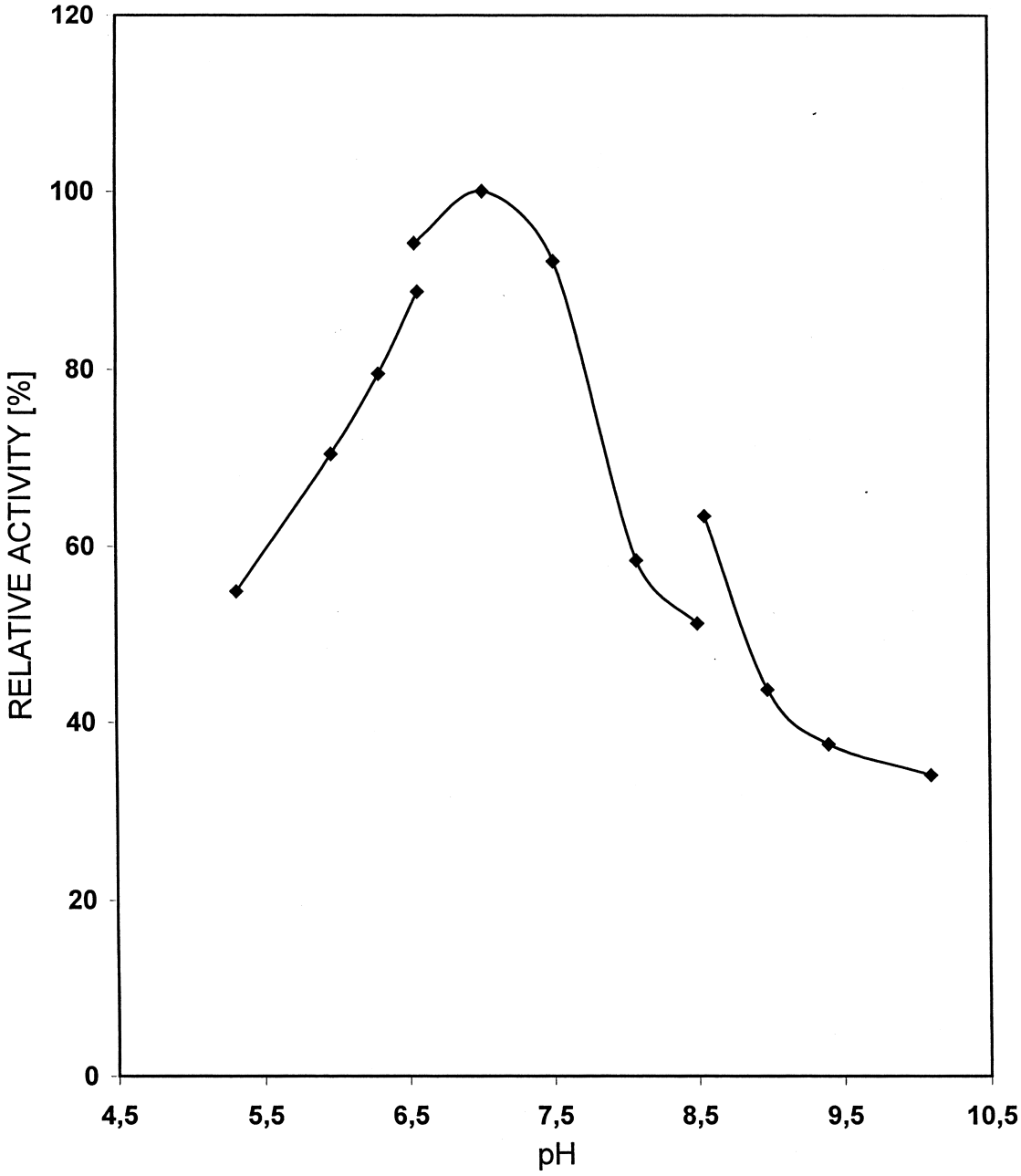

Fig. 6. pH dependence of the rhodanese-catalysed reaction. The reaction was performed using 50 m*M* MES buffer (pH 5.0–6.5), 50 m*M* HEPES buffer (pH 6.5–8.5) or 50 m*M* borate buffer (pH 8.5–10.0). Other conditions as in Fig. 3.

activity determined by a CZE method using the continuous spectrophotometric method (see above). The values obtained, i.e. 1.67 and 1.75 IU/ml, respectively, were in a good agreement.

The method developed was used to study some properties of the enzyme, such as the effects of pH and temperature on the enzymatic reaction. The experimentally determined pH dependence of the rhodanese-catalysed reaction is shown in Fig. 6. The experimental pH optimum agreed well with literature

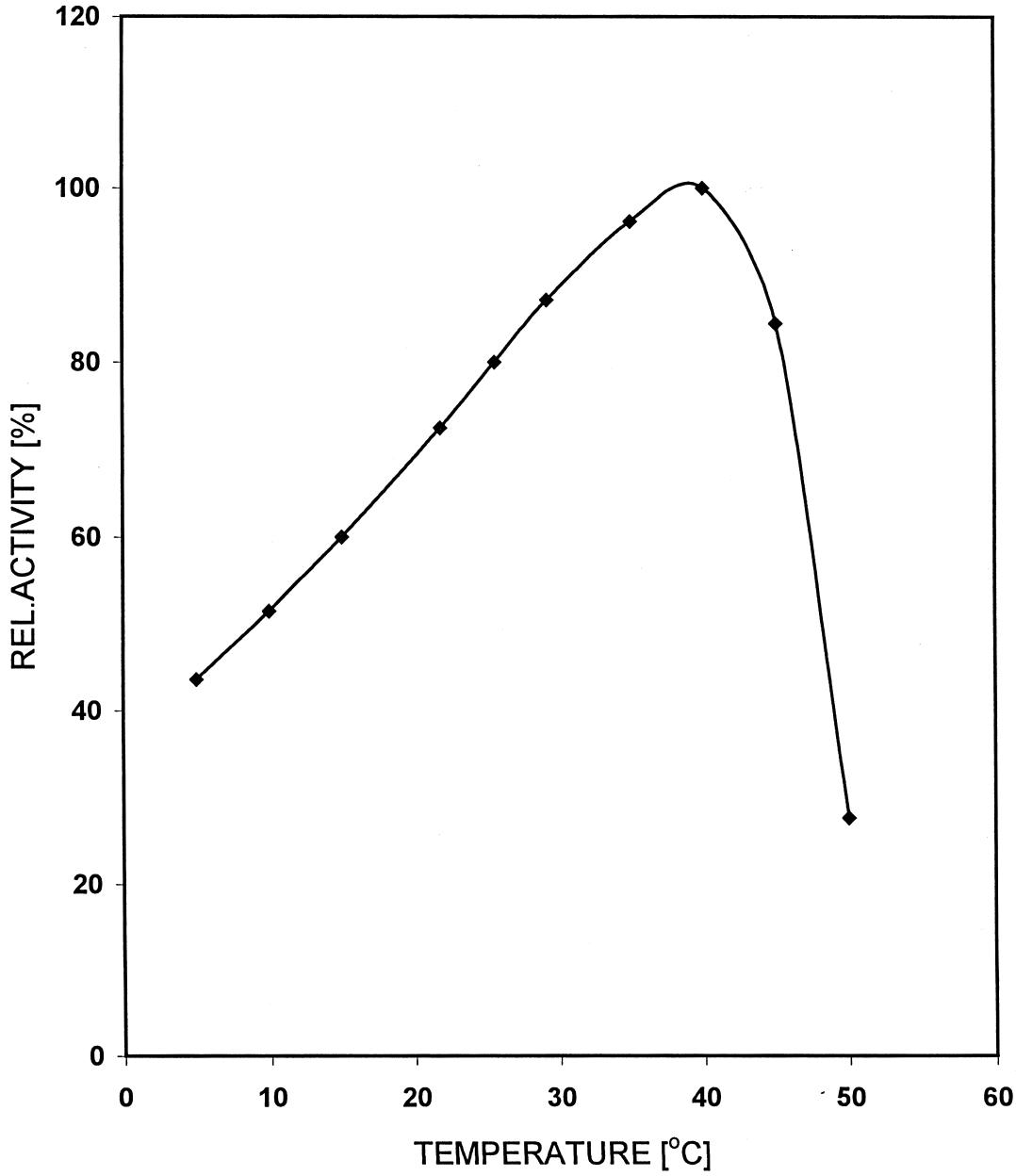

Fig. 7. Influence of temperature on the rhodanese-catalysed reaction. Reactions were performed at the temperature given, as described in Fig. 3.

values [14]. We also studied the influence of temperature on rhodanese activity, which is graphically illustrated in Fig. 7. An optimum was observed around 40°C, beyond which, rapid enzymatic denaturation occurred. The temperature optimum is also in good agreement with literature values [14].

4. Conclusion

A simple and rapid method for rhodanese activity has been developed. Compared to spectrophotometric and other assays, the capillary electrophoretic method is rapid, able to be automated and requires only small amounts of sample and substrates, which is especially important in the case of highly toxic cyanide. Moreover, the enzymatic reaction can be monitored with high sensitivity and reproducibility.

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