CHEMISTRY LETTERS, pp. 307-310, 1993. © 1993 The Chemical Society of Japan

On-line HPLC Determination of Enzymatic Activity of Alkaline Phosphatase in Natural Water Using Spectrofluorometric Detection

Akio HIROSE, Yukihiro ESAKA, Michiru OHTA, and Hiroki HARAGUCHI*
Department of Applied Chemistry, School of Engineering,
Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464

Fundamental studies on the on-line HPLC detection of enzymatic activity of alkaline phosphatase (APase) has been examined, where 4-methylumbelliferyl phosphate was used as a substrate. The technique developed was applied to detection of APase in natural water.

Alkaline phosphatase (APase) is contained in various kinds of organims such as human, animal, fish, bacteria etc.[1] and also in natural waters where such organisms are living.[2] It is further known that its enzyme detection or the chromatographic (or electrophoretic) pattern, as well as APase amounts, is useful for medical diagnosis of the animal organs.[3] In the case of natural water, the analysis of APase gives the interesting understandings for physicochemical behaviors in nature; for instances, the speciation for zinc in natural water [2,4] or the ecological mechanism of phosphorus cycles. [5]

In order to analyze APase in biological or environmental samples, the separation from other components is first required. In these studies HPLC (high performance liquid chromatography) has been generally employed, where two detecting principles of APase have been utilized. One is the method based on the UV-absorption of APase, and the other is that utilizing its enzymatic activity. From the viewpoint of the sensitivity as well as the selectivity, the latter is much superior to the former, as will be described later.

As the detection method of the enzymatic activity of APase, two methods have been reported. One is based on spectrophotometry [5] and the other on fluorometry.[6] In the present experiment, these two methods were examined to compare their sensitivities, using p-nitrophenyl phosphate (p-NPP) and 4-methylumbelliferyl phosphate (4-MUP) as the substrates. These substrates were dissolved in 0.6 M (M = mol/dm^3) Tris-HCl solution (pH 8.0) to be 0.1 mM. The results obtained by a batch method for Escherichia Coli APase (Sigma,

Product No.4151, enzymatic activity = 31 U/mg) are shown in Table 1. The detection limits in Table 1 were estimated as follows. In the case of spectrofluorometry, the detection limit was calculated as the amount of APase which gave the signal corresponding to twice the standard deviation of the blank signal, while in the case of spectrophotometry it was done as the amount to give the signal corresponding to S/N=2 (S; absorption signal intensity in absorbance, N; baseline noise of the blank signal). It can be seen from Table 1 that the spectrofluorometric detection gave better sensitivity by about 200-times than the spectrophotometric one.

Table 1. Comparison of the detection limits for APase by spectrophotometry and fluorometry in a batch method

Reaction time[a)]	Detection limit /ppb	
	Spectrophotometry (p-NPP)[b)]	Fluorometry (4-MUP)[b)]
3 min	1.1×10^{3}	5.3
1 h	55	0.26
24 h	2.3	1.1×10^{-2}

a) Incubation time. b) Substrate used.

The experiment was then extended to the on-line post-column HPLC detection. The experimental system used was similar to that shown in Fig. 1, except for the use of 0.2 mM p-NPP substrate dissolved in 0.1 M Tris-HCl solution as an eluting buffer and a shorter reaction tube of 7 m length (0.5 mm i.d.). In both cases of fluorometric and photometric detections, the detection limits were estimated according to S/N=2. The detection limits were calculated to be about 57 and 620 pg for the on-line fluorometric and photometric detections, respectively. Thus, in the on-line detection the fluorometric method gave better sensitivity by about 10-times than the photometric one, although 200-times of sensitivity difference was obtained in the batch method, as described above. Such poorer sensitivity in the on-line fluorometric detection was mainly caused by the less stable

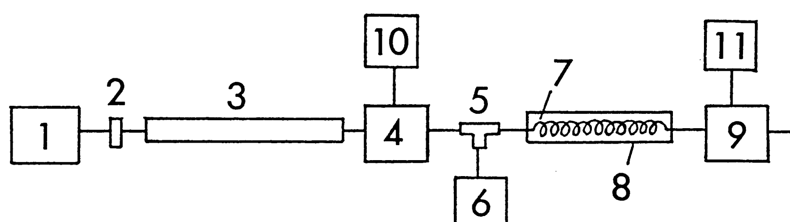

Fig. 1. Block diagram of the on-line HPLC system used for analysis of APase in natural water.
1. pump (eluent), 2. loop injector, 3. column, 4. UV-vis detector, 5. T-tube, 6. pump (4-MUP), 7. reactor (0.5 mm i.d. x 50 m), 8. thermostat, 9. fluorescence detector, 10,11. recorder.

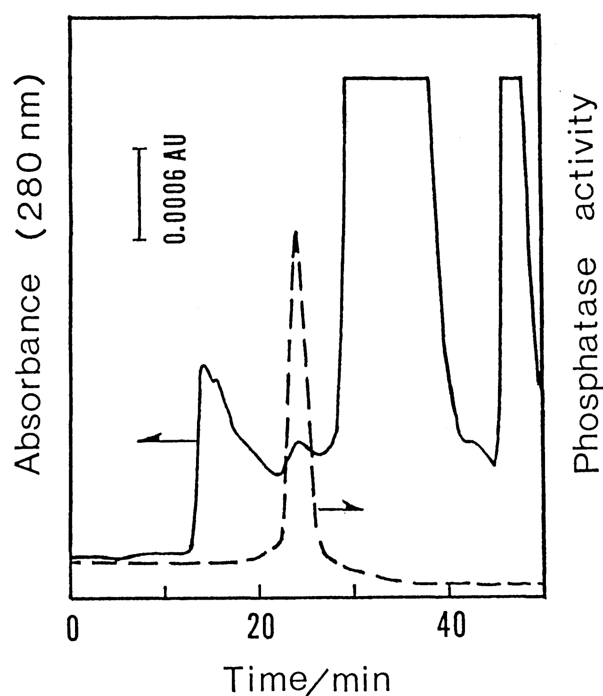

Fig. 2. SEC chromatograms for 160-fold concentrated pond water.
—: Spectrophotometric detection at 280 nm.
--- : Fluorometric detection of enzymatic activity.
Injection volume of sample: 1 ml.

baseline .

The analytical method for the on-line fluorometric detection of APase activity developed in the present experiment was applied to the analysis of natural water sample. The block diagram of the experimental system used is shown in Fig. 1. The FPLC system (Pharmacia-LKB) with a Superose 12 column of 30 cm x 10 mm i.d., a MV-7 valve injector and a LCC-500 Plus controller were used for size exclusion chromatography (SEC). Tris-HCl buffer solution (0.1 M) of pH 8 was used as an eluent at a flow rate of 0.5 ml/min. After SEC separation, the chromatograms were monitored at the 2 stages; first by a UV-monitor (LKB model 2141) at 280 nm to detect various kinds of organic compounds contained in natural water and then by a fluorometric one (JASCO model 812-FP) to detect APase activity using a 4-MUP substrate. The substrate was dissolved in an eluting buffer to be a 0.1 mM solution and added to column effluent through a T-tube at a flow rate of 0.1 ml/min. As a reaction tube the PTFE tubing of 0.5 mm i.d. x 50 m length was used, which was installed in a thermostat, although all chromatographic experiments were performed at room temperature. The fluorescence from 4-methylumbelliferone (4-MU) as the reaction product of 4-MUP was monitored at 362 and 442 nm as excitation and emission wavelengths, respectively. Before injecting a pond water sample, the sensitivities of this detection system for APase itself were compared, and it was found that the fluorometric measurement of enzymatic activity gave better sensitivity by about 700-times than the direct UV-detection of APase.

The natural water sample was then analyzed with the above system. Sample water was collected from the Kagami-ga-ike pond in the campus of Nagoya University, filtered through a 0.45 μm membrane filter (Advan-

tec), and concentrated by 160-fold with an ultrafiltration system (Advantec) using a 10,000-M.W. wet-type ultrafilter (43 mm i.d.) equipped in a UHP-43K stirred cell (70 ml volume), which was connected to a 1 l volume RP-1 reservoir. The chromatograms obtained are shown in Fig. 2, where the solid and broken lines are the chromatograms observed by an UV absorption and a fluorometric detections, respectively. It is seen in Fig. 2 that APase could be selectively detected by the fluorometric detection of the enzymatic activity, although it was not clear in the UV-monitored chromatogram.

The present on-line detection method using HPLC is rapid and convenient for APase enzymatic activity analysis, and thus it may be useful for the studies on APase in natural waters as well as in biological samples.

The present research was supported by a Grant-in-Aid for the Scientific Research (No.02453060) from the Ministry of Education, Science and Culture.

References

1)W. Meyer-Sabellek, P. Sinha, and E. Kottgen, *J. Chromatogr.*, **429**, 419 (1988).

2) K. Kobayashi, and H. Haraguchi, *Nippon Kaisui Gakkaishi*, **42**, 155 (1988).

3) T. Komoda, I. Koyama, and Y. Sakagishi, *Tanpakushitu Kakusan Kouso*, **30**, 1408 (1985).

4) K. Kobayashi, K. Fujiwara, and H. Haraguchi, *Kaiyo Kagaku*, **147**, 267 (1982).

5) K. Kobayashi, K. Iwase, S. Hashimoto, H. Ueda, K. Fujiwara, A. Otsuki, K. Fuwa, and H. Haraguchi, *Bull. Chem. Soc. Jpn.*, **60**, 925 (1987).

6) S. Hashimoto, K. Kobayashi, K. Fujiwara, H. Haraguchi, and K. Fuwa, *Bunseki Kagaku*, **32**, E177 (1983).

(Received October 22, 1992)