酵素センサによる海藻中の修酸の定量

誌名	日本水産學會誌
ISSN	00215392
著者	渡辺, 悦生 橋本, 務 今野, 敏徳
巻/号	55巻5号
掲載ページ	p. 911-915
発行年月	1989年5月

Nippon Suisan Gakkaishi 55(5), 911–915 (1989)

Determination of Oxalic Acid in Algae with an Enzyme Sensor

Etsuo Watanabe,*1 Tsutomu Hashimoto,*1 Toshinori Konno,*2
and Kenzo Toyama*1

(Received December 2, 1988)

Oxalic acid sensor was developed and applied to the determination of oxalic acid in some algae. The sensor was prepared with the combination of an oxalate oxidase membrane and an oxygen electrode. Oxalate oxidase was covalently immobilized on a membrane prepared from cellulose triacetate, 1, 8-diamino-4-aminomethyloctane and glutaraldehyde. The optimum operating condition of this sensor was determined as follows; pH: 6.0, temperature: 20°C, flow rate of buffer solution: 0.6 ml/min.

One assay could be completed within 3 min. The enzyme sensor could be used for more than 70 assays without the decrease of output current (standard deviation: 24 μM).

Algae were ground with mortar and treated with 85°C-hot water. After the water extracts were mixed with an equal amount of chroloform-methanol (1: 1), the water layers were used for the determination of oxalic acid. The results obtained by the sensor system were fairly agreeable with those of the conventional methods.

Oxalic acid is classified as a plant toxin. It is well-known that food poisoning is frequently caused by oxalic acid contained in foods. Therefore, oxalic acid used as a food additive is not permitted, of course, to be remained in food from the standpoint of food sanitation. The development of rapid and simple method for the determination of oxalic acid has been desired.

Oxalic acid has been recovered as calcium salt and determined by the titration method[1] or colorimetry.[2,3] And the acid has been also determined from carbon dioxide (1),[4] reduced nicotinamide (2),[5] or hydrogen peroxide (3)[6] formed in the following enzyme reactions:

$$(COOH)_2 \xrightarrow{\text{oxalate decarboxylase}} CO_2 + HCOOH \quad (1)$$

$$HCOOH + NAD \xrightarrow{\text{formate dehydrogenase}} C_2O + H_2O + NADH \quad (2)$$

$$(COOH)_2 \xrightarrow{\text{oxalate oxidase}} 2CO_2 + H_2O_2 \quad (3)$$

When the enzyme reaction (1) is applied for an oxalic acid enzyme sensor, the electrode used to measure CO_2 is usually unstable; while the procedures employing the enzyme reactions (2) and (3) are tedious and time-consuming.

In this paper, the oxalic acid sensor was developed by combining immobilized oxalate oxidase with an oxygen electrode. In addition, oxalic acids in some algae were determined by the proposed sensor.

Materials and Methods

Materials

Oxalate oxidase (EC 1.2.3.4) was purchased from Boehringer Mannheim Co. Oxalic acid, glutaraldehyde, 1, 8-diamino-4-aminomethyloctane and cellulose triacetate were obtained from Kokusan Kagaku Co., Tokyo Kasei Kogyo Co., Asahi Kasei Kogyo Co. and Eastman Kodak Co., respectively.

Preparation of Samples

The following algae were collected at Kominato in Chiba prefecture; *Sargassum siliquastrum*, *Sargassum macrocarpum*, *Sargassum patens*, *Eisenia bicyclis*, *Plocamium telfairiae*, *Hizikia fusiformis*, *Sargassum horneri*, *Gelidium amansii*, *Cladophora wrightiana*, *Carpopeltis angusta* were washed with water and dried at room temperature. Five g of each alga was ground with mortar, extreacted with 85°C-hot water for 1 h and centrifuged at 4000 rpm for 5 min. Each supernatant was diluted with water until the total volume of

*1 Department of Food Science and Technology, Tokyo University of Fisheries, Konan, Minato, Tokyo 108, Japan (渡辺悦生，橘本 務，外山健三：東京水産大学食品生産学科).

*2 Department of Aquatic Biosciences, Tokyo Univeristy of Fisheries, Konan, Minato, Tokyo 108, Japan (今野敏徳：東京水産大学資源育成学科).

the solution was 10 m*l*. In this sensor, 1 m*l* of each extract was further mixed with 2 m*l* of chroloform-methanol (1 : 1) solution and permitted to stand for 1 h. The supernatant was used for the analysis of oxalic acid. On the other hand, in the titration method and colorimetry, total of each hot water extract was mixed with 200 m*l* of saturated calcium hydroxide and neutralized with acetic acid. Oxalic acid was precipitated as calcium salt. The precipitate was dissolved with 5 m*l* of 1 N sulfuric acid and used for the analysis of oxalic acid.[1,2]

Preparation of Immobilized Oxalate Oxidase Membrane

About 10 sheets of membranes prepared from cellulose triacetate, glutaraldehyde and 1, 8-diamino-4-aminomethyloctane were immersed in a 0.1% (w/v) glutaraldehyde solution (0.05 M Tris-HCl buffer, pH 8.4) for 2 h at 30°C[7]). After washing with 0.05 M phosphate buffer (pH 7.8), they were placed in 1 m*l* of the same buffer containing oxalate oxidase (2 units) for 1 h at 30°C. The membranes were preserved in the same phosphate buffer at 5°C.

Apparatus and Assay Procedure

The sensor system was the same as that utilized for hypoxanthine,[7] inosine,[8,9] IMP[10] and glucose[11] determinations except for an immobilized enzyme membrane. The temperature and flow rate of 0.05 M phosphate buffer (pH 6.0) was maintained at 20°C and 0.6 m*l*/min, respectively, during the oxalic acid determination. When output current of the enzyme sensor reached a constant value, a sample solution containing oxalic acid was injected into the system and the current change was recorded. The maxium current decrease was used as a measure of oxalic acid concentration. An oxalic acid determination was also carried out by the conventional methods.[1,2]

Results and Discussion

Response Curve of the Enzyme Sensor

Fig. 1 depicts response curve of the oxalic acid sensor. After a current output was stabilized, a 50 μl aliquot of oxalic acid solution (3 mmol) was injected into the flow line. The oxygen was consumed as a result of the enzyme reaction. The output current began to decrease within 30 sec after the injection of sample and was restored to the original value by the passage of the sample through the cell after about 3 min. Therefore, the current decrease between the initial and the minimum currents was used as the measure of oxalic acid concentration.

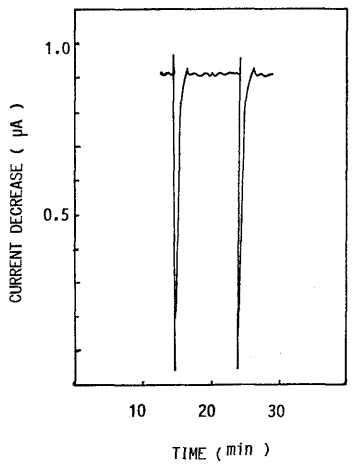

Fig. 1. Response curve of the enzyme sensor. Conditions; temperature: 20°C, flow rate: 0.6 m*l*/min, pH: 6.0, sample volume: 50 μl of 3.0 mmol oxalic acid.

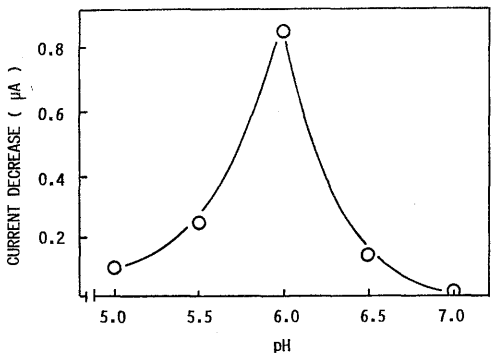

Fig. 2. Effect of pH on the current decrease of the sensor. Conditions; temperature: 20°C, flow rate: 0.6 m*l*/min, sample volume: 50 μl of 3.0 mmol oxalic acid.

Effect of Assay Conditions on Current Decrease

Effects of pH, temperature, flow rate and sample volume on responses of the enzyme sensor to oxalic acid were investigated.

Effect of pH: The optimum pH of natural oxalate oxidase is about 4.0.[12] But the the determination of the optimum pH of the immobilized oxalate oxidase is required for the establishment of the optimum conditions for the proposed sensor system. Therefore, the effect of pH on the current decrease was investigated. Fig. 2 shows the changes in current decrease for 3 mmol oxalic

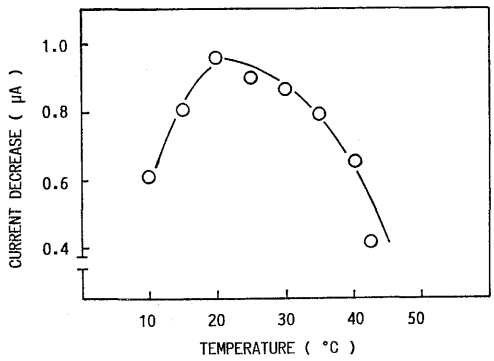

Fig. 3. Effect of temperature on the current decrease of the sensor. Conditions; flow rate 0.6 m*l*/min, sample volume: 50 μl of 3.0 mmol oxalic acid, pH: 6.0.

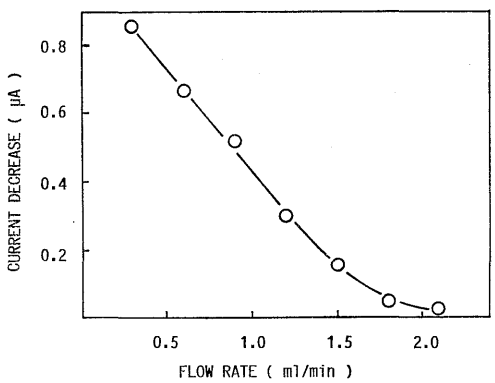

Fig. 4. Effect of flow rate on the current decrease of the sensor. Conditions: temperature: 20°C, pH: 6.0, sample volume: 50 μl of 3 mmol oxalic acid.

acid solution (50 μl) at various pH values. The optimum pH was found to be 6.0. Subsequent works were carried out at pH 6.0.

Effect of temperature: It is well-known that the activity of an enzyme depends on temperature. Therefore, the influence of temperature on the output of the enzyme sensor was also examined by the flow system at various temperatures (Fig. 3). The optimum temperature of the sensor toward oxalic acid was about 20°C. Therefore, a temperature of 20°C was employed in subsequent experiments.

Effects of flow rate and sample volume: The response of an enzyme sensor depends on the flow rate of a buffer solution and a sample volume in the flow analysis. Fig. 4 presents the influence of the buffer flow rate on the current decrease. The current decrease dropped with an increasing in flow rate, probably because of the incomplete

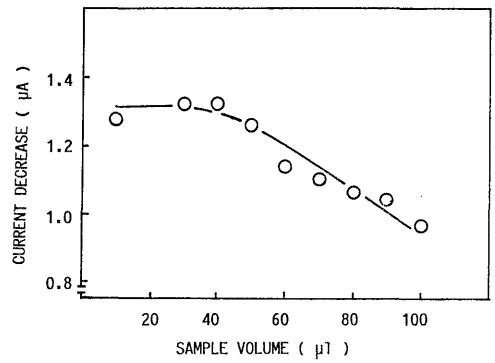

Fig. 5. Effect of sample volume on the current decrease of the sensor. Conditions; temperature: 20°C, pH: 6.0, flow rate: 0.6 m*l*/min, sample: 3.5 mM oxalic acid.

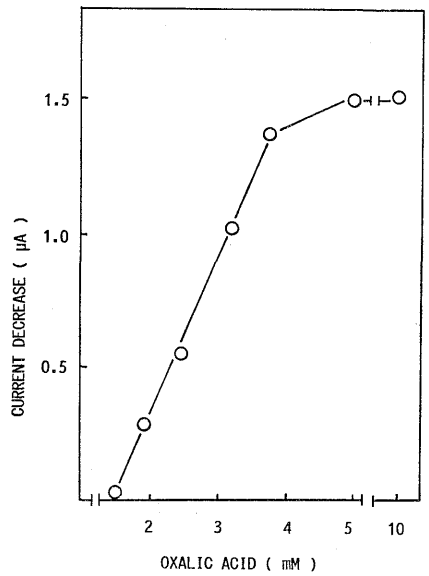

Fig. 6. Calibration curve for the oxalic acid sensor. Conditions: temperature: 20°C, pH: 6.0, flow rate: 0.6 m*l*/min, sample volume: 50 μl.

enzymatic reaction which occurred at this condition. A flow rate of 0.6 m*l*/min was employed in subsequent works. Fig. 5 gives the effect of sample volume on the response of the sensor. The current decrease was constant in the range 10–40 μl, while it gradually decreased above 50 μl. However, the sample volume was adjusted to 50 μl because the oxalic acid content in each algae was fairly low.

Calibration Curve for Oxalic Acid

The calibration curve of oxalic acid is shown in Fig. 6. One m*l* of each oxalic acid standard solution was mixed with 2 m*l* of chroloform-

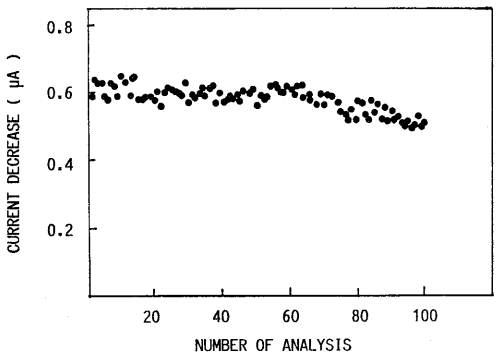

Fig. 7. Reusability of the oxalic acid sensor. Conditions; temperature: 20°C, pH: 6.0, flow rate: 0.6 ml/min, sample volume: 50 μl of 2.5 mM oxalic acid.

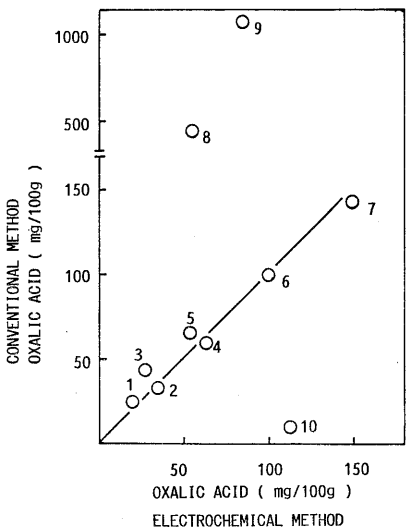

Fig. 8. Correlation between the oxalic acid concentrations of algae determined by the sensor and conventional methods. 1. *Sargassum siliquastrum*, 2. *Sargassum macrocarpum*, 3. *Sargassum patens*, 4. *Eisenia bicyclis*, 5. *Hizikia fusiformis*, 6. *Plocamium telfairiae*, 7. *Cladophora wrightiana*, 8. *Sargassum horneri*, 9. *Gelidium amansii*, 10. *Carpopeltis angusta*.

methanol (1 : 1) solution and permitted to stand for 1 h. 50 μl of the supernatant was injected at pH 6.0, 20°C, and 0.6 ml/min of flow rate. As given in Fig. 6, linearity of the curve was obtained between 1.5 and 3.7 mM. A partial inhibition of the enzyme by methanol could be the reason why the calibration curve did not pass the origin.

Reusability

The reusability of the sensor was examined using 50 μl sample solution containing 2.5 mM of oxalic acid. Under the assay conditions of pH 6.0, 20°C, and 0.6 ml/min, the standard deviation was ± 24 μM in 70 experiments (Fig. 7). However, the response of the sensor considerably decreased in more than 70 experiments.

Application of the Sensor for the Determination of Oxalic Acid in Algae

The contents of oxalic acid in some algae described above were determined by both the enzyme sensor and conventional methods. The results obtained were shown in Fig. 8. Within samples No. 1 to No. 7, good comparative results were obtained determined by the proposed flow system and the conventional method. However, the contents of oxalic acid in samples No. 8 and No. 9 were overestimated by the conventional method. This seemed to be due to the contamination of some reductive products besides oxalic acid. On the other hand, an amount of oxalic acid in sample No. 10 determined by the same method was less than that obtained by the enzyme sensor method. This could be the measurement error caused by the presence of colored substances in the sample solution.

Enzyme sensor method is based on the enzyme reaction which is specific to a particular substrate. Therefore, the isolation of an objective substance or decolorization of colored sample solution is dispensable in the determination of a substrate by an enzyme sensor. From the viewpoint mentioned above, the proposed sensor method for the determination of oxalic acid seemed to be superior to the conventional methods.

References

1) N. Fujii: Seikagaku Zikkenho, Nanzando Co., Tokyo, 1961, pp. 118–119.
2) J. Bergerman and J. S. Elliot: *Anal. Chem.*, **27**, 1014–1015 (1955).
3) K. Nakahara: *J. Jap. Soc. Nutri. Food Sci.*, **27**, 33–38 (1974).
4) R. K. Kobos and T. A. Ramsey: *Anal. Chim. Acta*, **121**, 11–000 (1980).
5) H. Yamanaka, M. Kuno, K. Shiomi, and T. Kikuchi: *J. Food Hyg. Soc. Japan*, **24**, 454–458 (1983).
6) R. Bais, N. Potezyny, J. B. Edwards, A. M. Rofe, and R. A. J. Conyers: *Anal. Chem.*, **52**, 508–511 (1980).
7) E. Watanabe, K. Ando, I. Karube, H. Matsuoka, and S. Suzuki: *J. Food Sci.*, **48**, 496–500 (1983).
8) E. Watanabe, K. Toyama, I. Karube, H. Matsuoka, and S. Suzuki: *Appl. Microbiol. Biotechnol.*,

19, 18–22 (1984).
9) E. Watanabe, H. Endo, T. Hayashi, and K. Toyama: *Biosensors*, **2**, 235–244 (1986).
10) E. Watanabe, K. Toyama, I. Karube, H. Matsuoka, and S. Suzuki: *J. Food Sci.*, **49**, 114–116 (1984).
11) E. Watanabe, H. Endo, Y. Ikeda, N. Shibamoto, and K. Toyama: *Nippon Suisan Gakkaishi*, **52**, 711–717 (1986).
12) M. F. Laker, A. F. Hofmann, and B. J. D. Meeuse: *Clin. Chem.*, **26**, 827–830 (1980).

Nippon Suisan Gakkaishi : Formerly *Bull. Japan. Soc. Sci. Fish.*