Spectrofluorometric Determination of Uric Acid and Glucose by Use of Fe(III)-Thiacalix[4]arenetetrasulfonate as a Peroxidase Mimic

Junichi Odo,*,[a] Eri Shinmoto,[a] Atsushi Shiozaki,[a] Yoko Hatae,[a] Seiichi Katayama,[a] and Geng-sheng Jiao[b]

[a]Department of Biological Chemistry, Faculty of Science, Okayama University of Science, 1–1 Ridai-cho, Okayama 700–0005, Japan and [b]Department of Chemistry, Weinan Teachers College, Weinan, Shaanxi 714000, China

(Received June 11, 2004; Accepted August 19, 2004)

The peroxidase-like activity of the Fe^{3+} complex of thiacalix[4]arenetetrasulfonate on a modified anion-exchanger (Fe^{3+}-TCAS[4]$_{A\text{-}500}$) has been applied to the spectrofluorometric determination of uric acid and glucose in connection with uricase and glucoseoxidase (GOD), respectively. Uric acid and glucose were spectrofluorometrically determined by measuring the amounts of H_2O_2 produced through each catalytic reaction by uricase and GOD for uric acid and glucose in a sample solution, respectively. The calibration curves obtained by the Uricase-Fe^{3+}-TCAS[4]$_{A\text{-}500}$ and the GOD-Fe^{3+}-TCAS[4]$_{A\text{-}500}$ methods were linear from 0.5 to 5.0 μg of uric acid in a 1.0 ml sample solution and from 0.5 to 6.0 μg of glucose in a 0.5 ml sample solution, respectively. The methods using Fe^{3+}-TCAS[4]$_{A\text{-}500}$ were applied to the determination of uric acid and glucose in control sera.

Key words ——— spectrofluorometric determination, uric acid, glucose, thiacalix[4]arene, peroxidase-like activity, modified anion-exchanger

INTRODUCTION

In clinical analyses many enzymatic methods are routinely used to measure various vital compounds.[1] For example, uric acid and glucose are indirectly determined by measuring the amounts of H_2O_2 produced through reactions (1) and (2) catalyzed by uricase and glucoseoxidase (GOD), respectively. In general, the amount of H_2O_2 produced was spectrophotometrically determined by measuring an amount of quinoid dye produced through reaction (3) catalyzed by peroxidase (POD).

$$\text{uric acid} + O_2 + 2H_2O \xrightarrow{\text{uricase}} \text{allantoin} + H_2O_2 + CO_2 \quad (1)$$

$$\text{glucose} + O_2 \xrightarrow{\text{GOD}} \text{gluconolactone} + H_2O_2 \quad (2)$$

$$\text{chromogen} + 2H_2O_2 \xrightarrow{\text{POD}} \text{quinoid dye} + 4H_2O \quad (3)$$

However, most enzymes generally have problems regarding stability, handling and storage. In an effort to develop an artificial mimesis to solve these problems, we have investigated the enzyme-like activities of metal complexes of thiacalix[4]arenetetrasulfonate on a modified anion-exchanger (Me-TCAS[4]$_{A\text{-}500}$).[2–5] We have shown that Fe^{3+}-TCAS[4]$_{A\text{-}500}$ exhibited strong peroxidase-like activity for reaction (3) and was useful as a peroxidase mimic for the spectrophotometric determination of H_2O_2 and glucose in control sera.[2,3] Moreover, we have demonstrated that Fe^{3+}-TCAS[4]$_{A\text{-}500}$ exhibited strong catalytic activity for oxidation (4) of *p*-acetoamidophenol to produce fluorescent substances in the presence of H_2O_2 (It is well known that *p*-hydroxypheny derivatives such as *p*-acetoamidophenol are oxidized to produce a fluorescent dimer by peroxidase in the presence of H_2O_2. Accordingly, the catalytic reaction for *p*-acetoamidophenol by Fe^{3+}-TCAS[4]$_{A\text{-}500}$ was estimated by referring to the

*To whom correspondence should be addressed: Department of Biological Chemistry, Faculty of Science, Okayama University of Science, 1–1 Ridai-cho, Okayama 700–0005, Japan. Tel. & Fax: +81-86-256-9429; E-mail: odo@dbc.ous.ac.jp

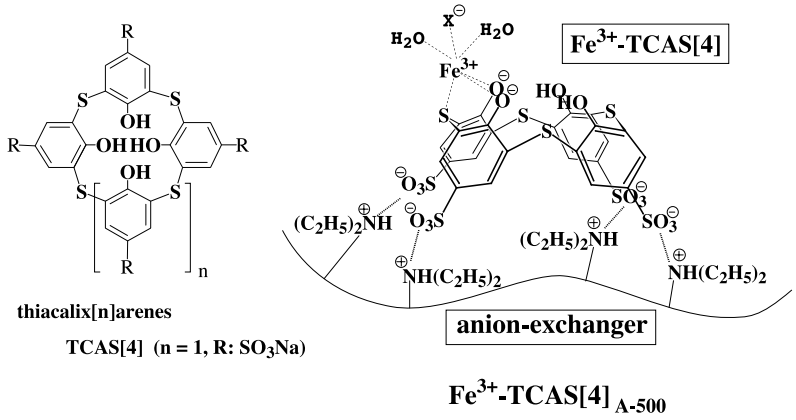
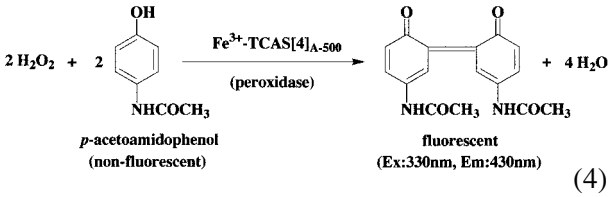


Fig. 1. Proposed Structure of Fe^{3+}-TCAS[4]$_{A\text{-}500}$

corresponding reaction by peroxidase.), and was useful as a peroxidase mimic for the spectrofluorometric determination of a trace amount of H_2O_2.[5)]

In clinical analyses, it is necessary to develop a more sensitive method for the determination of vital compounds so as to minimize the amount of vital samples. In this study, the peroxidase-like catalytic activity of Fe^{3+}-TCAS[4]$_{A\text{-}500}$ for oxidative reaction (4) was applied to the spectrofluorometric determination of uric acid and glucose in connection with uricase and GOD, respectively. To date, spectrofluorometric methods using various enzymes have been developed for the determination of uric acid[6)] and glucose.[7–10)] However, no report has been published on the spectrofluorometric method using a peroxidase mimic such as Fe^{3+}-TCAS[4]$_{A\text{-}500}$.



(4)

MATERIALS AND METHODS

Reagents —— Sodium thiacalix[4]arenetetrasulfonate (TCAS[4], Fig. 1) was kindly provided by Cosmo Oil Co. (Tokyo, Japan). DEAE cellulofine A-500 (an anion-exchanger of cellulose-type with diethylaminoethyl groups) purchased from Seikagaku Kogyo Co. (Tokyo, Japan). was washed with water several times and dried over P_2O_5 under reduced pressure. *p*-Acetoamidophenol from Nakalai Tesque Co. (Kyoto, Japan). was recrystallized from hot water several times. Peroxidase (POD, from horseradish), and control serum I (normal) and II (abnormal) were purchased from Wako Pure Chemical Industries Co. (Osaka, Japan). Glucoseoxidase (GOD, from *Aspergillus niger*), uricase (from *Candida sp.*) and ascorbate oxidase (from *Cucurbita sp.*) were purchased from Sigma-Aldrich Japan Co. (Tokyo, Japan). All other chemicals were of analytical or reagent grade and used without further purification.

Fe^{3+}-TCAS[4]$_{A\text{-}500}$ (Fig. 1) was prepared according to the literature.[5)] Fe^{3+}-TCAS[4]$_{A\text{-}500}$ contained 100 μmol of Fe^{3+}-TCAS[4] per g of dry anion-exchanger.

The buffer solutions used were 0.1 M CH_3COOH–0.1 M CH_3COONa for pH 5.0, 0.1 M KH_2PO_4–0.05 M $Na_2B_4O_7$ for pH 6.5–9.0, 0.1 M NH_4Cl–0.1 M NH_4OH for pH 8.5–11.0, and 0.1 M $NaHCO_3$–0.05 M Na_2CO_3 for pH 9.0–11.5.

Instruments —— The fluorescence intensities were recorded on a Shimadzu RF-5300 spectrofluorometer with a quartz cell (1 × 1 cm cross-section) equipped with a xenon lamp and dual monochromator.

Procedure ——

Uricase-Fe^{3+}-TCAS[4]$_{A\text{-}500}$ Method for Uric Acid: Uric acid was determined with a combination of reactions (1) and (4) catalyzed by uricase and Fe^{3+}-TCAS[4]$_{A\text{-}500}$, respectively. The fluorescence intensity was measured with excitation and emission wavelengths of 330 and 430 nm, respectively.

Fe^{3+}-TCAS[4]$_{A\text{-}500}$ (20 mg) was added to a mixture containing sample solution (1.0 ml, 0.5–5.0 μg of uric acid), uricase solution (1.0 ml, 1.25 U/ml), 40 mM *p*-acetoamidophenol solution (1.0 ml) and

buffer solution (3.0 ml); the mixture was incubated at 40°C for 30 min. After Fe^{3+}-TCAS[4]$_{A\text{-}500}$ was filtered off, the fluorescence intensity of the supernatant was measured.

Uricase-POD Method for Uric Acid as a Reference: A mixture of sample solution (1.0 ml), uricase solution (1.0 ml, 1.25 U/ml), POD solution (0.5 ml, 10 U/ml), 40 mM *p*-acetoamidophenol solution (1.0 ml) and buffer solution (2.5 ml) was incubated at room temperature for 30 min. The fluorescence intensity of this reaction solution was measured.

GOD-Fe^{3+}-TCAS[4]$_{A\text{-}500}$ Method for Glucose: Glucose was determined with a combination of reactions (2) and (4) catalyzed by GOD and Fe^{3+}-TCAS[4]$_{A\text{-}500}$, respectively. First, a mixture containing sample solution (0.5 ml, 0.5–6.0 μg of glucose), GOD solution (0.5 ml, 32 U/ml), and acetate buffer solution of pH 5.0 (0.5 ml) was incubated at 40°C for 10 min. Next, 40 mM *p*-acetoamidophenol solution (2.0 ml), carbonate buffer solution of pH 10.25 (2.5 ml) and Fe^{3+}-TCAS[4]$_{A\text{-}500}$ (20 mg) were added and the mixture was incubated at 40°C for 20 min. After Fe^{3+}-TCAS[4]$_{A\text{-}500}$ was filtered off, the fluorescence intensity of the supernatant was measured.

GOD-POD Method for Glucose as a Reference: A mixture of sample solution (0.5 ml), GOD solution (0.5 ml, 32 U/ml), POD solution (0.5 ml, 10 U/ml), 40 mM *p*-acetoamidophenol solution (2.0 ml) and buffer solution (2.5 ml) was incubated at room temperature for 20 min. The fluorescence intensity of this reaction solution was measured.

Removal of Serum Proteins from Control Sera: A mixture of serum (2.0 ml), 2/3 N H_2SO_4 (1.0 ml) and 10 g/dl sodium tungstate (1.0 ml) was vigorously shaken for 10 min and then centrifuged at 3000 rpm for 10 min. The supernatant (1.0 ml) of this reaction mixture was used as a sample solution.

RESULTS AND DISCUSSION

Investigation of Optimum Conditions for the Determination of Uric Acid

For the determination of uric acid, uricase (from *Candida sp.*) and Fe^{3+}-TCAS[4]$_{A\text{-}500}$ were simultaneously added to a sample solution. However, the optimum pH for each of their activities differed slightly. The optimum pH of uricase (from *Candida sp.*) is about 8, whereas Fe^{3+}-TCAS[4]$_{A\text{-}500}$ exhibited its maximum catalytic activity for reaction (4) at around pH 10, as described previously.[5] Accordingly, to establish the optimum conditions, experiments were carried out using the Uricase-Fe^{3+}-TCAS[4]$_{A\text{-}500}$ method with 4 μg of uric acid.

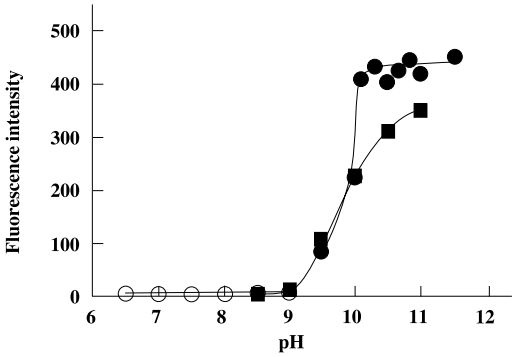


Fig. 2. Effects of the pH and Buffer on the Uricase-Fe^{3+}-TCAS[4]$_{A\text{-}500}$ Method
●, $NaHCO_3$–Na_2CO_3; ○, KH_2PO_4–$Na_2B_4O_7$; ■, NH_4Cl–NH_4OH.

Effects of pH

The activity of the Uricase-Fe^{3+}-TCAS[4]$_{A\text{-}500}$ was investigated in various buffer solutions. Figure 2 shows the fluorescence intensities of the solutions obtained through reactions (1) and (4) by uricase and Fe^{3+}-TCAS[4]$_{A\text{-}500}$ in each solution. As previously described,[5] Fe^{3+}-TCAS[4]$_{A\text{-}500}$ exhibited its maximum peroxidase-like activity for oxidative reaction (4) at around pH 10. As shown in Fig. 2, Uricase-Fe^{3+}-TCAS[4]$_{A\text{-}500}$ exhibited high levels of activity in alkali buffer solutions, especially in a carbonate buffer solution of pH 10–11.5. Accordingly, a carbonate buffer solution of pH 10.5 was selected for the determination of uric acid with the Uricase-Fe^{3+}-TCAS[4]$_{A\text{-}500}$ method.

Effects of the Concentration of *p*-AP and Incubation Time

The concentration of *p*-AP and the incubation time affected the Uricase-Fe^{3+}-TCAS[4]$_{A\text{-}500}$ method. Forty mM of *p*-AP was the optimum concentration of the fluorogenic reagent: the fluorescence intensities were almost maximum and constant over 30 mM of *p*-AP, as shown in Fig. 3. Figure 4 shows that the fluorescence intensities were near maximal and constant after 20 min of incubation. Thus, 30 min of incubation was long enough.

The Calibration Curve

Under the optimum conditions described above, a linear calibration curve was obtained between 0.5

and 5.0 μg of uric acid in a sample solution (1.0 ml) using the Uricase-Fe^{3+}-TCAS[4]$_{A\text{-}500}$ method. The correlation coefficient and the relative standard deviation (n = 4) were 0.981 and 5.5% for 4 μg of uric acid in a sample solution (1.0 ml), respectively. Accordingly, the Uricase-Fe^{3+}-TCAS[4]$_{A\text{-}500}$ method is applicable to the determination of a trace amount of uric acid.

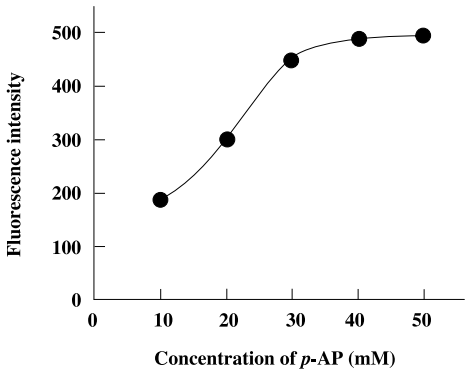


Fig. 3. Effect of the Concentration of p-AP on the Activity of the Uricase-Fe^{3+}-TCAS[4]$_{A\text{-}500}$ Method

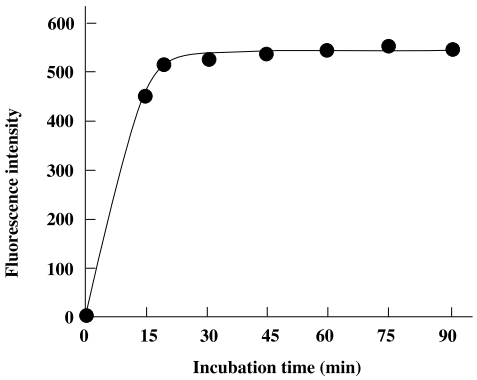


Fig. 4. Effect of the Incubation Time on the Uricase-Fe^{3+}-TCAS[4]$_{A\text{-}500}$ Method

Effect of Foreign Substances

Interference in the Uricase-Fe^{3+}-TCAS[4]$_{A\text{-}500}$ method was investigated in the presence of various foreign substances. As shown in Table 1, ascorbate showed severe interference, and human serum albumin (HSA) and Fe^{3+} ion also had an appreciable interference. Although HSA caused almost no interference in the Fe^{3+}-TCAS[4]$_{A\text{-}500}$ method to determine H_2O_2,[5] it did affect the Uricase-Fe^{3+}-TCAS[4]$_{A\text{-}500}$ method. It is not clear why the interference by HAS was different between the present Uricase-Fe^{3+}-TCAS[4]$_{A\text{-}500}$ technique and the Fe^{3+}-TCAS[4]$_{A\text{-}500}$ technique[5] for the determination of H_2O_2. The severe interference by ascorbate may be caused by reaction of ascorbate with H_2O_2 produced through reaction (1) and/or by reduction of Fe^{3+}-TCAS[4]$_{A\text{-}500}$ by ascorbate to Fe^{2+}-TCAS[4]$_{A\text{-}500}$, which showed low peroxidase-like activity.[2] Also, the interference of Fe^{3+} ion may be caused by reaction of Fe^{3+} ion with H_2O_2. Accordingly, the addition of ascorbate oxidase and EDTA-2Na (disodium dihydrogen ethylenediaminetetraacetate) to a sample solution should minimize the interference from ascorbate and Fe^{3+} ion, respectively. As shown in Table 1, the interference from these ions was markedly decreased by adding 0.5 ml of ascorbate oxidase solution (10 U/ml) and EDTA-2Na solution (7 mM) to a sample solution (1.0 ml), respectively.

Table 1. Effect of Foreign Substances on the Uricase-Fe^{3+}-TCAS[4]$_{A\text{-}500}$ Method to Determine Uric Acid (UA)

Substance	Added (μg)	Error (%)[a]	Substance	Added (μg)	Error (%)[a]
Heparin	12.1 (I.U.)	1.9	Fe^{3+}	40	−27.4
NaF	40	−9.5		40	−11.4[c]
EDTA	40	−6.6	K^+	40	0.9
Glycine	40	6.6	Ca^{2+}	40	−14.7
Ascorbate	40	−81.2	PO_4^{3-}	40	3.4
	40	−7.2[b]	CO_3^{2-}	40	0.5
Citrate	40	3.0	NH_4^+	40	−3.7
Albumin (HSA)	10^4	−37.4	Br^-	40	1.7
			I^-	40	−2.3

UA added; 4.0 μg. *a*) Error (%) = 100X(UA(found) − UA(added))/UA(added). *b*) Ascorbate oxidase was added to the sample solution. *c*) EDTA-2Na was added to the sample solution.

Application to the Determination of Uric Acid in Sera

The Uricase-Fe^{3+}-TCAS[4]$_{A\text{-}500}$ method was applied to determine uric acid in control serum I (normal) and II (abnormal). As this technique was affected by HSA, the sample solution after the removal of serum proteins in control sera was applied to the determination. As shown in Table 2, the observed amounts of uric acid obtained with the Uricase-Fe^{3+}-TCAS[4]$_{A\text{-}500}$ method were just a little lower than those with the Uricase-POD method as a reference. This may be the reason why the simultaneous reaction efficiency of uricase and Fe^{3+}-TCAS[4]$_{A\text{-}500}$ is not always adequate. However, the results with the present method were satisfactory for both control serum I and II.

Investigation of Optimum Conditions for the Determination of Glucose

Similar to uric acid, glucose was determined by measuring the fluorescence intensity of the reaction solution through reactions (2) and (4) catalyzed by GOD and Fe^{3+}-TCAS[4]$_{A\text{-}500}$, respectively. However, it was difficult to use both GOD (from *Aspergillus niger*) and Fe^{3+}-TCAS[4]$_{A\text{-}500}$ in the same sample solution, because their optimum pHs are quite different, unlike the case of uricase and Fe^{3+}-TCAS[4]$_{A\text{-}500}$. Thus, Fe^{3+}-TCAS[4]$_{A\text{-}500}$ exhibited strong catalytic activity for reaction (4) in a carbonate buffer at around pH 10, whereas the optimum pH of GOD (from *Aspergillus niger*) is around 5. Accordingly, GOD and Fe^{3+}-TCAS[4]$_{A\text{-}500}$ were added independently to the sample solution. After GOD was added to this solution in acetate buffer of pH 5.0 and the mixture was incubated for 10 min, Fe^{3+}-TCAS[4]$_{A\text{-}500}$ was added in a carbonate buffer of pH 10.25. The optimum conditions for reaction (4) catalyzed by Fe^{3+}-TCAS[4]$_{A\text{-}500}$, for example, the pH of the sample solution, concentration of *p*-AP, and incubation temperature and time, were the same as those[5] for the determination of H_2O_2 using Fe^{3+}-TCAS[4]$_{A\text{-}500}$. The calibration curve was straight for sample solutions (0.5 ml) containing 0.5–6.0 μg of glucose, and the correlation coefficient and relative standard deviation ($n = 4$) were 0.985 and 4.9% for 5.0 μg of glucose, respectively. The sensitivity of the present method was 10–100 times higher than those of the spectrophotometric methods we previously developed using Fe^{3+}-TCAS[4]$_{A\text{-}500}$.[2,3]

Moreover, the GOD-Fe^{3+}-TCAS[4]$_{A\text{-}500}$ method was applied to the determination of glucose in control sera I and II, whose sample solutions were applied after being diluted 20 and 100 times with distilled water, respectively. This technique required no removal of serum proteins such as HSA from control sera. This may be because the concentration of HSA decreased substantially on dilution of the sera. As shown in Table 3, the observed amounts of glucose obtained for both control serum I and II were satisfactory, compared with the GOD-POD method as a reference.

In conclusion, the peroxidase-like activity of Fe^{3+}-TCAS[4]$_{A\text{-}500}$ for reaction (4) was useful for the spectrofluorometric determination of a trace amount of uric acid and glucose in connection with uricase and GOD, respectively. Moreover, these methods using Fe^{3+}-TCAS[4]$_{A\text{-}500}$ were also useful for the spectrofluorometric determination of a trace amount of uric acid and glucose in blood. Fe^{3+}-TCAS[4]$_{A\text{-}500}$ has the advantages that it can be prepared easily and used repeatedly, and exhibits sufficient activity as a peroxidase mimic even in sera. The present methods are the first successful examples performed by a peroxidase mimic, Fe^{3+}-TCAS[4]$_{A\text{-}500}$, for the spec-

Table 2. The Amounts of Uric Acid in Serum I and II after Removal of Serum Protein Determined by the Uricase-Fe^{3+}-TCAS[4]$_{A\text{-}500}$ and the Uricase-POD Methods

Method	Uric acid (μg/1.0 ml)	
	serum I	serum II
Uricase-Fe^{3+}-TCAS[4]$_{A\text{-}500}$	39.9	77.2
Uricase-POD	44.3	85.1

Table 3. The Amounts of Glucose in Diluted Serum I and II Determined by the GOD-Fe^{3+}-TCAS[4]$_{A\text{-}500}$ and the GOD-POD Methods

Method	Glucose amount (μg/0.5 ml)	
	serum I (diluted 20 times)	serum II (diluted 100 times)
GOD-Fe^{3+}-TCAS[4]$_{A\text{-}500}$	19.4	13.2
GOD-POD	18.6	11.6

trofluorometric determination of uric acid and glucose.

Acknowledgements The authors thank Cosmo Oil Co. for supplying sodium thiacalix[4]arenetetrasulfonate. Thanks are also due to Drs. H. Kumagai and S. Miyanari of Cosmo Oil Co. for supporting this work.

REFERENCES

1) Kanai, M., Okabe, H., Sekiguchi, M., Nomoto, S., Kameko, M., Isobe, M., Tozuka, M., Hidaka, H., Oguchi, M. and Kawa, S. (1998) Clinical chemistry examination (in Japanese). In *Rinsyo-Kensaho-Teiyo* (Kanai, I. and Kanai, M., Eds.), Kinbara Press. Tokyo, chap. 6, pp. 459–682.
2) Odo, J., Kawahara, N., Inomata, Y., Inoue, A., Takeya, H., Miyanari, S. and Kumagai, H. (2000) Peroxidase-like catalytic activity of metal complexes of thiacalix[4]arenetetrasulfonate on the modified ion-exchanger and its application for the determination of hydrogen peroxide. *Anal. Sci.*, **16**, 963–966.
3) Odo, J., Inomata, Y., Takeya, H., Miyanari, S. and Kumagai, H. (2001) Determination of hydrogen peroxide by Iron(III) complex of thiacalix[4]arenetetrasulfonate on a modified ion-exchanger with peroxidase-like catalytic activity. *Anal. Sci.*, **17**, 1425–1429.
4) Odo, J., Yamaguchi, H., Ohsaki, H. and Ohmura, N. (2004) Catalytic activity for decomposition of hydrogen peroxide by metal complexes of water-soluble thiacalix[4]arenetetrasulfonate on the modified anion-exchangers. *Chem. Pharm. Bull.* (Tokyo), **52**, 266–269.
5) Odo, J., Matsumoto, K., Shinmoto, E., Hatae, Y. and Shiozaki, A. (2004) Spectrofluorometric determination of hydrogen peroxide based on oxidative catalytic reactions of *p*-hydroxyphenol derivatives with metal complexes of thiacalix[4]arenetetrasulfonate on a modified anion-exchanger. *Anal. Sci.*, **20**, 707–710.
6) Kamoun, P., Fargis, F., Hebert, C. and Lafourcade, G. (1976) Automated ultramicromethod for plasma uric acid determination. *Ann. Biol. Clin.*, **34**, 387–391.
7) Sanz, V., Galban, J., de Marcos, S. and Castillo, J. R. (2003) Fluorometric sensors based on chemically modified enzymes. Glucose determination in drinks. *Talanta*, **60**, 415–423.
8) Matsu-ura, S., Yamauchi, Y., Ohmori, H. and Maeda, H. (2002) Blood glucose determination with the reduction of resazurin as a fluorometric indicator reaction. *Bunseki Kagaku.*, **51**, 111–115.
9) Stoll, R. W. and Wen, S.-F. (1978) A simple fluorometric method for glucose determination in nanoliter samples. *Kidney Int.*, **14**, 191–193.
10) Mori, H., Ishida, M. and Okamoto, S. (1999) Glucose determination using a flow system with enzyme reactor and application to analysis of glucose content in beverages. *J. Health Sci.*, **45**, 126–129.