J. Flow Injection Anal., Vol. 8, No. 2 (1991)

FLOW-INJECTION MICRODETERMINATION OF HEAVY METAL IONS USING A COLUMN PACKED WITH IMMOBILIZED APOENZYME BEADS

IKUO SATOH

Department of Chemical Technology, Faculty of Engineering,
Kanagawa Institute of Technology,
1030 Shimo-Ogino, Atsugi-shi, 243-02, Kanagawa-ken, Japan

ABSTRACT

A novel idea for biochemical microanalysis of heavy metal ions in combination with flow-injection techniques is proposed. The microdetermination of the cofactors based on an apoenzyme reactivation method is reviewed exemplifying the assays for heavy metal ions with use of several kinds of immobilized metalloenzymes as the recognition elements for each metal ion.

Key words : alkaline phosphatase, apoenzyme, ascorbate oxidase, biosensing, biosensor, calorimetry, carbonic anhydrase, cofactor, cobalt(II) ions, copper(II) ions, enzyme column, flow-injection analysis, galactose oxidase, heavy metal ions, immobilzed enzyme, metalloenzyme, microanalysis, reactivation, regeneration, zinc(II) ions.

1. INTRODUCTION

During the past quarter century numerous kinds of biosensors using biospecific materials have been developed[1]. Currently, around twenty bioanalyzers including enzyme sensors are commercially available. However, most of the analytes have

been likely to be limited to any of substrates, products, inhibitors and activators in the enzymatic reactions so far. In contrast we have proposed a novel bioanalytical method for flow-injection microdetermination of cofactors based on an apoenzyme reactivation[2-15]. Many kinds of enzymes essentially need cofactors, e.g. nucleotides and metal ions for their catalytic reaction. In an acitve site of a metalloenzyme molecule heavy metal ions are coordinated and thereby, they can function as a cofactor in the catalysis. The metal ions are usually intrinsic to each of metalloenzymes. Therefore, the metal-free enzymes can be regarded as the specific recognition elements for each metal. Thus, we have tried to use cofactor-free enzymes regenerated from metalloenzymes as recognition elements for heavy metal ions in flow streams.

In this paper, the overview of the apoenzyme-reactivation flow-microassay of heavy metal ions is described focussing on our research works.

2. PRINCIPLE AND CHARACTERISTICS

2.1 Principle

Heavy metal ions or nucleotides are coordinated in the active site of the metalloenzymes or flavin enzymes and then, these enzymes are capable of expressing their catalytic activity. The cofactor-bound enzymes are generally called holoenzymes, while cofactor-free enzymes are apoenzymes. The interconversion process between the holoenzyme and the apoenzyme is mostly reversible as schematically illustrated in Fig.1. The metalloenzymes bind metal ions so tightly(dissociation constant: K_d<10^{-8}M) that the metal ions remain with the enzymes throughout the course of purification. On the other hand metal-activated enzymes associate metal ions loosely(K_d:10^{-3} to 10^{-8}M) and then, the latter metal ions dissociate from the metal-complexed enzymes during purification[17].

The apoenzyme lacking in its catalytic activity can be generated by removing the metal from the holoenzyme with strong

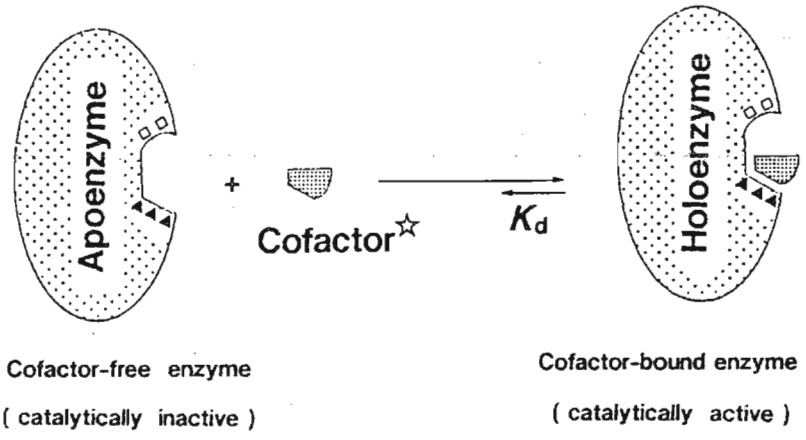

Fig.1 The complexing of the cofactor to the apoenzyme.

☆ : cofactor(heavy metal ion or nucleotide),□□: substrate-binding site,
▲▲▲: catalytic site.

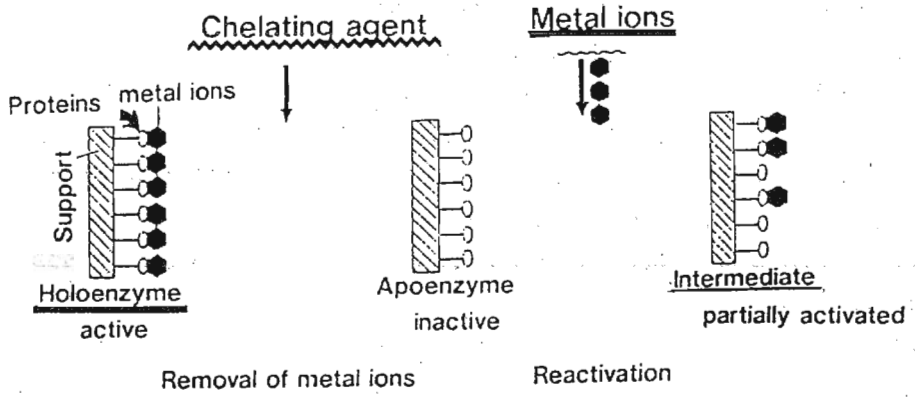

Fig.2 The interconversion process between the regeneration and the reactivation of the immobilized apoenzymes

chelating agents. The apoenzyme is reversibly reactivated by exposure to the metal-containing sample so that metal ions can be taken up and trapped in the active site. The amount of the metals complexed in the catalytic center of the enzyme molecules, therefore, may be related to the enzyme activity induced by the coordination and in turn proportional to the added amount of the metals. Thus, the trace metal content can be evaluated through measuring the expressed activity due to the reactivation of the apoenzyme.

The metal ions complexed in the active site of the metalloenzymes differs from the kind of the enzymes. Consequently, selective determination of the metals can be performed by applying the appropriate metalloenzymes in which their catalytic sites fit in well with each of the metals.

Use of a packed column containing immobilized metalloenzymes can make assays continuous and enhance feasibility of handling in the process between the regeneration and the reactivation of apoenzymes. The interconversion process is schematically shown in Fig.2. Reusability and long-term stability of the immobilized enzymes may be also expected. Microassay of heavy metal ions using spectrophotometric monitoring was reported by Townshend[18] and microdetermination with use of high performance liquid chromatographic method was also presented by Johansson[19] based on the similar idea, respectively. Mattiasson presented an electrochemical determination using an apoenzyme electrode[20].

We have originally developed biosensing of heavy metal ions in combination with flow-calorimetry[2]. Pioneer works on thermometric flow-injection analysis of biorelated componds using a high performance semiadiabatic calorimeter and a small column packed with immobilized enzymes have been made by Mosbach and Danielsson[21,22]. The measuring system is a kind of thermal bioanalyzers and is called "Enzyme thermistor". We applied the intrument to monitor the enzymatic activity in the reaction cycle including the regeneration and the reactivation. The schematic illustration of a calorimetric biosensing system(so to say, "Apoenzyme thermistor") is represented in Fig.3. The enzyme column is interchangeable and thereby, different species of

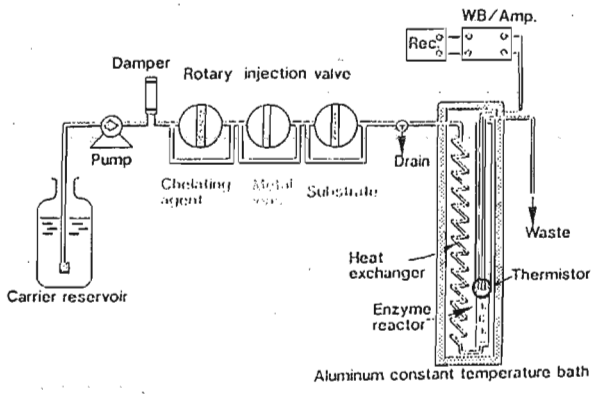

Fig.3 Schematic diagram of a flow-calorimetric biosensing system based on the apoenzyme reactivation method.

Pump(flow rate:1.00 ml min^{-1}), Bath(80 Φ x 250mm; 273 $\pm$ 0.0001K), Heat exchanger(thin-walled acid-proof steel tubing:0.8mm,i.d.), Enzyme reactor (packed with metalloenzymes immobilized onto beads), Thermistor(attached to a gold capillary placed in a polymer tube), WB/Amp.(DC-type Wheatstone bridge with a chopper stabilized operational amplifier).

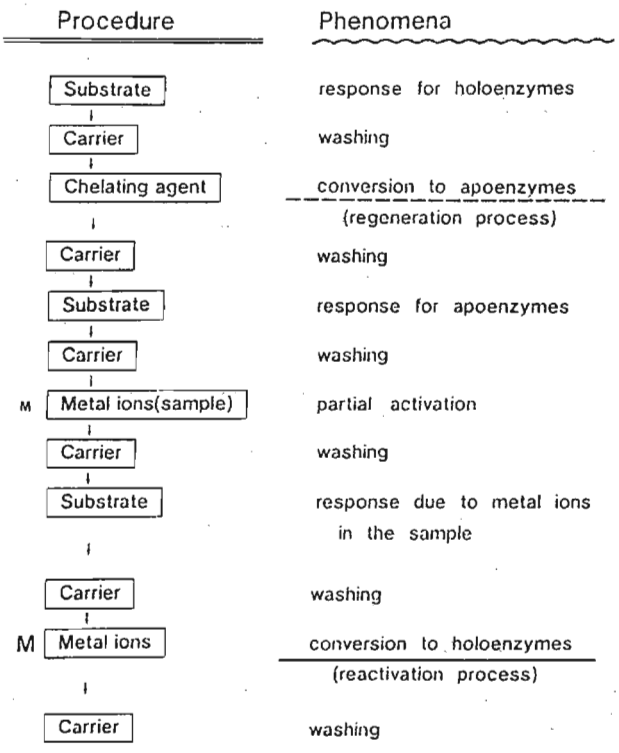

Fig.4 The flow-assay procedure based on the proposed method.

metals are very easily determined. Furthermore, other monitoring methods based on electrochemical and spectrophotometric determinations are available by exchanging the thermistor with other devices such as electrodes and photomultipliers.

2.2 Characteristics

Table 1 summarizes the characteristics of the proposed biosensing methods. The novel sensing methods in microassay of heavy metal ions don't need any expensive instruments based on atomic absorption spectrophotometry and inductively coupled plasma atomic emission spectrophotometry. In addition the proposed method is free from pollution problems due to exhaust fumes.

3. ASSAY PROCEDURE

The assay procedure proposed here is schematically illustrated in Fig.4. Three rotary injection valves are incorporated into the flow system not to be contaminated each other. At first the catalytic activity of the reactor packed with the immobilzed holoenzymes is monitored by injecting its substrate solution. A sharp peak obtained in a calorimetric system is a measure of the activity due to the holoenzymes as shown in Fig. 5. After exposing the cofactor-complexing agent to the reactor, a significant decrease in the activity is observed. This means most of the cofactors coordinated in the catalytic site of the holoenzymes are removed. Regeneration volumes of the reactor are dependent on kinds of metalloenzymes and furthermore conditions for the cofactor-complexing. Subsequent addition of a trace amount of cofactors reactivates the enzymes once chelator-exposed. Then, the recovery of the reactor activity is demonstrated by introducing the substrate. After sufficient cofactors have been injected to the system, the mixture of the cofactor-free and cofactor-bound enzymes can be converted to the completely reactivated enzymes. Thus, the system is ready for another assay. A reaction cycle usually takes 40 min to 60 min.

Table 1 Characteristics of the Flow-injection Microdetermination of Heavy Metal Ions Using a Column Packed with Immobilized Apoenzyme Beads

Mild assay conditions
High sensitivity
High selectivity
Reusability of the enzymes due to the immobilization
Considerable loading capacity of the enzymes in small space due to the use of porous supporting materials
Low-priced assay
Continuous flow use
Compact assay system
Feasibility of handling
Feasibility of field works
Separability of metal-trapping and activity-monitoring process
Versatility due to the interchangeability of the enzyme reactor

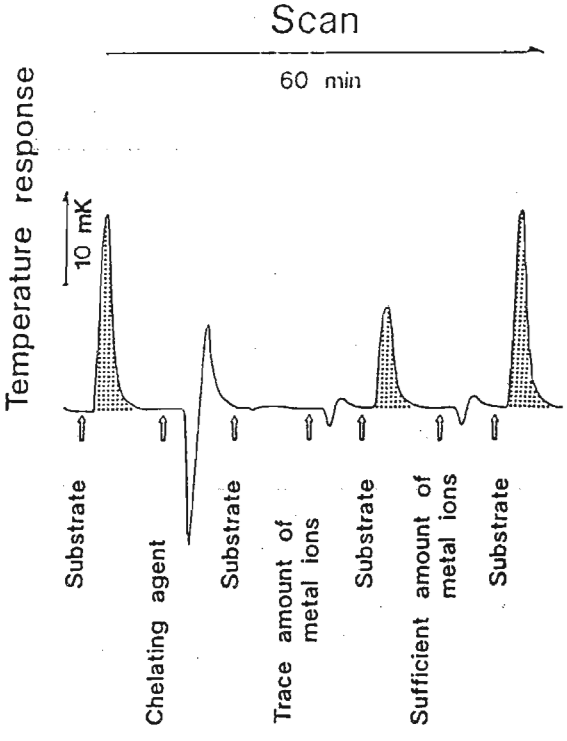

Fig.5 Schematic presentation of response curves for a reaction cycle in the flow-calorimetric biosensing of heavy metal ions.

4. BIOSENSING OF HEAVY METAL IONS

4.1 Biosensing of zinc(II) ions

4.1.1 Assay with carbonic anhydrase

There are so many zinc-dependent metalloenzymes known so far[23]. Carbonic anhydrase possesses one gram-atom of zinc per mol. The enzyme purified from erythrocytes(EC 4.2.1.1) has very high turnover numbers and plays a very important role controlling equilibrium between carbon dioxide and bicarbonate in bloods.

$$CO_2 + H_2O \xrightarrow[\textit{anhydrase}]{\textit{Carbonic}} H_2CO_3 \qquad (1)$$

The enzyme also reveals an esterase activity as shown in equation(2). Bovine carbonic anhydrase(EC 4.2.1.1) was immobilized onto porous glass beads with controlled pore size and then applied to specific determination of zinc(II) ions[2].

$$p\text{-Nitrophenyl acetate} + H_2O \xrightarrow[\textit{anhydrase}]{\textit{Bovine carbonic}} p\text{-nitrophenol} + \text{acetate} \qquad (2)$$

Injection of 0.5 ml of *p*-nitrophenyl acetate solution of various concentrations into the system with the holoenzyme reactor(packed volume: 0.3 ml) gave an exothermic response. A linear relationship with a slope of 2.7 mK M^{-1} was obtained between the concentrations of the substrate and the changes in temperature response. Application of the tris-HCl(pH 8.0) buffer as the carrier provided three times higher responses than those of phosphate buffer(0.9 mK M^{-1}). Normally values of the changes in enthalpy(ΔH) for ester hydrolysis reactions are zero. Therefore, this exothermic change was mainly due to the protonizing heat of acetic acid enzymatically released to the tris carrier solution. The relative standard deviation of thirty repeated measurements of 3 mM substrate was about 2%. The

cofactor-bound enzyme reactor retained 83% of the initial activity under the condition of 303K and pH 8.0 during 60 days.

Addition of 10 mM 2,6-pyridine dicarboxylate solution(pH 5.0) as the cofactor-complexing agent to the enzyme reactor sufficiently saturated with zinc(II) ions caused a marked decrease in the temperature response owing to the protonizing heat. The residual activity after exposure to more than 5ml with the chelating agent was almost not observed. The effect of the pH in the chelating agent on the regeneration was examined and no remarkable decrease in the enzyme activity was shown in the basic region. Zinc(II)ions in the range from 25 μM to 250 μM could be determined for 0.5 ml injections.

4.1.2 Assay with alkaline phosphatase

Use of alkaline phosphatase enabled much highly sentitive determination of zinc(II) ions based on a couple of monitoring methods[9-11,16] and also exhibited a considerably greater long-term stability than that of the method using the immobilized bovine carbonic anhydrase. The enzyme is often used as a label for enzyme immunoassays[24]. The following reaction is normally utilized for monitoring the enzyme activity.

$$p\text{-Nitrophenyl phosphate} + H_2O \xrightarrow[\textit{phosphatase}]{\textit{Alkaline}} p\text{-nitrophenol} + \text{orthophosphate} \quad (3)$$

Alkaline phosphatase(from *Escherichia coli* ; EC 3.1.3.1) immobilized onto epoxide acrylic beads(Eupergit C: 100-200 μm particle diameter; 40 nm pore diameter; 180 m^2g^{-1} surface area; Röhm Pharma, Darmstad, Germany) was packed into a small column(0.29 ml) and then used as the recognition element.

Tris-HCl 100 mM buffer(pH 8.0, containing 1.0 M NaCl) was used as the carrier solution and the catalytic activity was calorimetrically monitored by injecting 0.1 ml of 100 mM substrate solution(*p*-nitrophenyl phosphate). Exposing 20 mM 2,6-pyridine dicarboxylate solution(pH 6.0) as the chelating

agent to the enzyme reactor preliminary saturated with zinc(II) ions caused a considerable decrease in the temperature response due to the protonizing heat of phosphoric acid generated by the enzyme-catalyzed reaction. Complete regeneration was virtually achieved by introduing 2.5 ml of the chelator to the reactor. The reactor maintained almost constant catalytic activity before and after the regeneration process. The effect of the pH in the chelator on the regeneration was investigated for 0.1 to 0.5 ml injections in the pH range of 4.0 to 8.0 and no appreciable difference in the level of the sufficient regeneration was observed over the pH region.

The recovery of the temperature response due to the partially reactivated apoenzyme was obtained as a function of the added amount of zinc(II) ions. Calibration graph for zinc(II) ions showed a sigmoid curve as illustrated in Fig.6. Zinc(II) ions in the range from 0.01 to 1.0 mM could be ultimately determined for 0.5 ml injections. The effect of pH in zinc(II) ions solution on the reactivation of the apoenzyme was examined in the weakly acidic pH region(from 4.0 to 6.0 in step of 0.5). The effect turned out to be almost invariable at each pH value. Therefore, the proposed determination of zinc(II) does not require a pretreatment of critical pH adjustment and is quite practical. The reactor has been repeatedly used for the regeneration/reactivation process over 120 times during the 2 months of operation.

Spectrophotometric monitoring of the enzyme activity measured by detecting change in absorbance at 405 nm due to the *p*-nirophenol formed gave similarly sensitive biosensing of zinc(II) ions in the submicromolar levels[9,10]. Potentiometric approach using the enzyme reactor in combination with a flow-through ISFET was also made by sensing change in pH due to phosphoric acid released in the enzymatic hydrolysis[10,11]. Thus, zinc(II) ions were potentiometrically determined in a range of 0.01 to 1.0 mM through the similar assay cycle.

4.2 Biosensing of copper(II) ions

4.2.1 Assay with ascorbate oxidase

Most of copper-depending enzymes are involved in catalytic

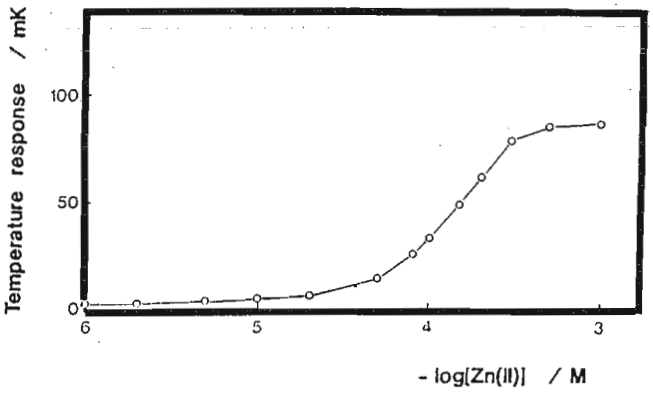

Fig.6 Calibration curve for zinc(II) ions with use of the flow-calorimetric biosensing system.

Carrier: 100 m M Tris-HCl(pH 8.0.,containing 1 M NaCl), Reactor: packed with the immobilized alkaline phosphatase(0.29ml), Sample volume: 0.5 ml, Substrate: 100 m M *p*-nitrophenyl phosphate(0.1 ml), Chelating agent: 20 mM 2,6-pyridine dicarboxylate(pH 6.0).

Table 2 Flow-injection Microdetermination of Heavy Metal Ions Based on Apoenzyme Reactivation Methods

Metal	Recognition element	Monitoring method/device		Range[mM]
Zn(II)	*Alkaline phosphatase*	Calorimetry	T	0.010 - 1.0
Zn(II)	*Alkaline phosphatase*	Potentiometry	I	0.010 - 1.0
Zn(II)	*Alkaline phosphatase*	Spectrophotometry	P	0.0001 - 0.010
Zn(II)	*Bovine carbonic anhydrase*	Calorimetry	T	0.025 - 0.25
Zn(II)	*Carboxypeptidase A*	Calorimetry	T	0.1 - 0.5
Cu(II)	*Ascorbate oxidase*	Amperometry	O	0.0005 - 0.002
Cu(II)	*Ascorbate oxidase*	Calorimetry	T	0.001 - 0.05
Cu(II)	*Ascorbate oxidase*	Spectrophotometry	P	0.0001 - 0.010
Cu(II)	*Galactose oxidase*	Amperometry	O	0.1 - 10.0
Cu(II)	*Galactose oxidase*	Amperometry	H	0.01 - 10.0
Cu(II)	*Galactose oxidase*	Calorimetry	T	5.0 - 20.0
Co(II)	*Alkaline phosphatase*	Calorimetry	T	0.04 - 1.0
Co(II)	*Alkaline phosphatase*	Spectrophotometry	P	0.001 - 0.2
Co(II)	*Bovine carbonic anhydrase*	Calorimetry	T	0.005 - 0.2

Device: T(thermistor), I(pH-ISFET), P(photomultiplier), O(polarographic oxygen electrode), H(hydrogen peroxide electrode).

oxidation. Ascorbate oxidase is one of the typical copper-enzymes and catalyzes oxidation of $_{L}$-ascorbate to dehydroascorbate as follows:

$$2\,_{L}\text{-Ascorbate} + O_2 \xrightarrow[\text{oxidase}]{\textit{Ascorbate}} 2\text{dehydroascorbate} + 2H_2O \qquad (4)$$

Oxidative reactions involving molecular oxygen are always accompanied by a large amount of heat evolution. Ascorbate oxidase from cucumber immobilized onto porous glass beads was applied to the proposed calorimetric system and the catalytic reaction was monitored. Then, considerable heat evolution was observed with injection of $_{L}$-ascorbate solution[25]. The results was to be anticipated for highly sentitive and precise microassay of copper(II) ions from the much exothermic response by using the immobilized enzymes as the recognition elements.

As was anticipated, micromolar levels of copper(II) ions could be determined.[3]. In particular, the element was not responsive to divalent cations in 1 mM level such as Ca(II), Co(II), Mg(II), Ni(II) and zinc(II) at all. Regeneration of the apoenzymes could be achieved by exposing the packed column to 1 ml of 20 mM *N,N*-diethyl dithiocarbamate solution(pH 8.0). One assay cycle took around 40 min. The calorimetric method was applied to trace assay of copper in human blood sera[26] and compared with the conventional atomic absorption spectrophotometric method. There was satisfactory agreement between both methods.

Furhtermore, amperometric monitoring of the reaction using an polarographic oxygen electrode was also separately performed and then, more sentitive assay for copper(II) ions(0.5 to 2.0 uM) could be realized[4]. The electrochemical sensing method was positively applied to determine the copper(II) content in cocoa powder.

In addition, the amount of $_{L}$-ascorbate consumed during the reaction was traced by the measurement of decrease in absorbance

at 265 nm. Copper(II) ions determined photometrically ranged from 0.1 to 10.0 μM. Thus, the validity and practical use of microdetermination of copper(II) ions using immobilized ascorbate oxidase as the recognition element were clarified.

4.2.2 Assay with galactose oxidase

Galactose oxidase(EC 1.1.3.9) has one gram atom of copper per mol and catalyzes the following reaction.

$$\text{D-Galactose} + O_2 \xrightarrow[\text{oxidase}]{\text{Galactose}} \text{D-}\textit{galacto}\text{-hexodialdose} + H_2O \quad (5)$$

The apoenzyme from *Dactilium dendroides* can be readily obtained under the condition of the water-soluble state, but it takes longer time to reactivate the apoenzyme. The calorimetric biosensing with use of the immobilized enzymes onto porous glass beads gave less sensitive and time-consuming assay of copper(II) ions, since remarkable heat evolution was not accompanied[12]. On the contrary, electrochemical methods using an oxygen and a hydrogen peroxide electrode as a monitoring device reduced the measuring time, and yet the lower detection limit of the methods were 0.1 mM[8]. Therefore, the less sensitivity may arise in not the transducers per se, but the characteristics of the enzyme element.

4.3 Biosensing of cobalt(II) ions

4.3.1 Assay with carbonic anhydrase

Zinc(II) ions coordinated in the catalytic site of some zinc-enzymes can be reversibly substituted with cobalt(II) ions and then the cobalt-complexed enzymes express still their catalytic activity. We tried to exchange the cobalt(II) ions with zinc(II) ions to be trapped in the active site of the bovine carbonic anhydrase[5]. Regeneration of a reactor packed with the immobilized carbonic anhydrase was positively made by exposing to less volume of 2,6-pyridine carboxylate solution(pH

6.0, 2.5 ml). The calibration in the calorimetric monitoring covered a range from 50 to 200 μM.

4.3.2 Assay with alkaline phosphatase

Cobalt-substituted alkaline phosphatase can also retain the estarase activity. The alkaline phosphatase immobilized on oxirane-acrylic beads were also applicable to the flow-injection photometric biosensing of cobalt(II) ions[15]. Cobalt(II) ions were determined in 1.0 to 200 μM. Repeated use of the reactor packed with the cobalt-substituted enzymes via regeneration/reactivation process performed at 303K has been possible over 230 times during 3 months of operation. Flow-calorimetric determination of cobalt(II) using the same immobilized preparations as the sensing elements was separately performed and thereby, the calibration range from 0.04 to 1.0 mM was monitored.

5. CONCLUSIONS

Flow-injection microdetermination of heavy metal ions based on apoenzyme reactivation method with use of immobilized metalloenzymes beads was demonstrated. Table 2 summarizes the results. The versatile and sensitive measurements of the enzyme activity could be achieved with calorimetric monitoring of the heat evolution due to the reactivated apoenzymes. The concept of the proposed method is readily applied to microassay of heavy metal ions in any analytical fields. Selective determination of heavy metals may be led by choosing appropriate metalloenzymes as the recognition element, in which its corresponding metal ion is complexed in its catalytic site.

Further developmental studies are currently in progress toward establishing the generality and versatility of the analitical techniques with use of immobilized apoenzyme system in flow streams.

REFERENCES

1. *Biosensors: Fundamentals and Applications*, eds., A.P.F. Turner, I. Karube and G. Wilson, Oxford University Press, New York (1988).
2. I. Satoh, K. Ikeda and N. Watanabe, *Proc. 6th Sensor Sym.*, ed., K. Takahashi, Institute of Electrical Engineers of Japan, Tokyo, Japan, pp.203-206(1986).
3. I. Satoh, S. Kimura and T. Nambu, *Digest of Technical Papers 4th Int. Conf. Solid-State Sensors and Actuators(Transducers '87)*, ed., T. Matsuo, Institute of Electrical Engineers of Japan, Tokyo, Japan, pp.789-792(1987).
4. I. Satoh, R. Abe and T. Nambu, *Denki Kagaku*, 56, 1045-1049 (1988).
5. I. Satoh, *Proc. MRS Int. Meet. Advanced Materials*, in *Biosensors*, Vol.14, Material Research Society, Pittsburgh, pp.45-50(1989).
6. I. Satoh, Kagaku Kogyo(in Japanese), 40, 423-428(1989).
7. I. Satoh, In *Chemical Sensor Technology*, Vol.2, ed., T. Seiyama, Kodansha Ltd., Tokyo, pp.269-282(1989).
8. I. Satoh, T. Kasahara and N. Goi, *Sensors and Actuators*, B1, 499-503(1990).
9. I. Satoh and T. Masumura, *Technical Digest 9th Sensor Sym.*, ed., A. Sasaki, Institute of Electrical Engineers of Japan, Tokyo, Japan, pp.197-200(1990).
10. I. Satoh, *Proc. 3rd Int. Meet. Chem. Sensors*, ed., C.C. Liu, Cleveland, USA, pp.P106-P107(1990).
11. I. Satoh and Y. Aoki, *Denki Kagaku*, 58, 1114-1118(1990).
12. I. Satoh, *Ann. N.Y. Acad. Sci.*, 613, 401-404(1990).
13. I. Satoh, *Netsusokutei(Calor. Therm. Anal.)*(in Japanese), 18, 89-96(1991).
14. I. Satoh and T. Nambu, *Technical Digest 10th Sensor Sym.*, ed., T. Nakamura, Institute of Electrical Engineers of Japan, Tokyo, Japan, pp.77-80(1991).
15. I. Satoh and Y. Yamada, *Digest Technical Papers 6th Int. Conf. Solid-State Sensors and Actuators(Transducers '91)*, ed., S. Chang, Institute of Electrical Engineers, Inc.,

Piscataway, pp.699-702(1991).
16. I. Satoh, *Biosensors and Bioelectronics*, 6, 375-379(1991).
17. F.W. Wagner, In *Methods in Enzymology*, Vol.158, eds., J.F. Riordan and B.L. Vallee, Academic Press Inc., San Diego, USA, pp.21-32(1988).
18. A. Townshend and A. Vaugham, *Talanta*, 17, 289-298(1970).
19. L. Riesinger, L. Ögren and G. Johansson, *Anal. Chim. Acta*, 154, 251-257(1983).
20. B. Mattiasson, H. Nilsson and B. Olsson, *J. Appl. Biochem.*, 1, 377-384(1979).
21. B. Danielsson and K. Mosbach, In *Methods in Enzymology*, Vol.137, ed., K. Mosbach, Academic Press Inc., New York and London, pp.181-197(1988).
22. B. Danielsson, *J. Biotechnol.*, 15, 187-200(1990).
23. B.L. Vallee, *Carlsberg Res. Commun.*, 45, 423-441(1980).
24. B.K. van Weemen and A.H.W.M. Schuurs, In *Principles of Enzymatic Analysis*, ed., H.U. Bergmeyer, Verlag Chemie, Weinheim and New York, pp.93-98(1978).
25. I. Satoh, M. Kawasaki and S. Sugawara, unpublished data.