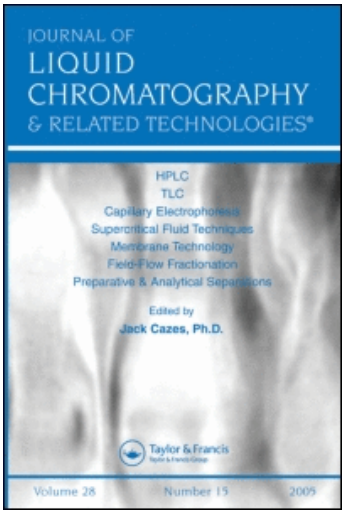

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DETERMINATION OF VANADIUM, COBALT, NICKEL, AND IRON IN BROMOPEROXIDASES FROM *PSEUDOMONAS PUTIDA* AND *CORALLINA PILULIFERA* BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH SPECTROPHOTOMETRIC DETECTION

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ABSTRACT

A highly sensitive, selective, and simple method for the determination of vanadium, cobalt, nickel, and iron has been proposed for the analysis of the enzymes. The method is based on the preliminary chelation of the metal ions with 2-(5-bromo-2-pyridylazo)-5-(diethylamino)phenol at pH 4.5 and the subsequent separation by reversed-phase high performance liquid chromatography using a trimethyl-bonded phase column and an aqueous acetonitrile containing tetrabutylammonium bromide, and spectrophotometric detection at 595 nm. The detection limits, defined as three times standard deviation of a blank signal, are 2.2 pg of vanadium, 0.72 pg of cobalt, 16.5 pg of nickel and 98.8 pg of iron in 100 mm^3-injection, respectively.

Vanadium, cobalt, nickel, and iron in the enzymes can be successfully determined without preseparation and preconcentration.

The proposed method is evaluated by comparing the analytical results with those obtained by inductively coupled plasma (ICP) atomic emission spectrometry and ICP mass spectrometry.

INTRODUCTION

Biological halogenation catalyzed by haloperoxidases has environmental, agricultural and pharmacological significance through their roles in the biosynthesis of volatile halogenated compounds,[1] antibiotics, and hormones.[2] So far, the enzymes catalyzing the halogenation have been considered heme iron-containing peroxidases.[3-5] However, a class of haloperoxidases discovered in marine algae[6,7] and fungi[8] has contained vanadium which has been identified by ESR.[9,10] Moreover, Itoh et al. found bromoperoxidase from *Pseudomonas putida* which has been activated by the incubation with cobalt ion.[11]

Thus, because some metal ions such as iron, vanadium, and cobalt function as the active site, the detection and the determination of the trace metals are of importance for clarifying the mechanism of the enzymatic reactions, and further investigation of metal enzymes will depend on the availability of a highly sensitive method for the metal determination.

Recently, reversed-phase high performance liquid chromatography (RP-HPLC) with spectrophotometric detection has been accepted as a powerful method for trace metal determinations.[12] One of the most convenient HPLC methods for metals is the pre-column derivatization method which is based on the preliminary chelation of the metal ions with a suitable chromogenic reagent such as azo dye compounds and the subsequent separation by HPLC and spectrophotometric detection.

2-(5-bromo-2-pyridylazo)-5-(diethylamino) phenol (5-Br-PADAP), among many azo dyes, is excellent in sensitivity for detection of metal ions, which has been employed for the simultaneous determination of vanadium, cobalt, nickel, and iron in high-purity silicon.[13] In this report, the HPLC method using 5-Br-PADAP is applied to the enzyme analysis.

EXPERIMENTAL

Reagents

2 x 10^{-3} M 2-(5-bromo-2-pyridylazo)-5-(diethylamino)phenol (5-Br-PADAP) solution was prepared as follows: a 0.175-g portion of 5-Br-PADAP (Dojindo Laboratories, Kumamoto, Japan) was dissolved into a 25-g portion of poly(oxyethylene) 4-nonylphenyl ether with 10 oxyethylene units (PONPE-10) (Tokyo Chemical Industry, Tokyo, Japan) with warming on a water bath and stirring; after the solution was diluted with about 150 cm^3 of water with warming and gently stirring, the resultant solution was transferred into a 250-cm^3 calibrated flask and made up to the mark with water. The surfactant, PONPE-10 was employed for solubilization of 5-Br-PADAP and its chelates. Standard solutions of metal ions were 100 mg dm^{-3} or 1000 mg dm^{-3} solution for atomic absorption spectrometry (Kanto Chemical, Tokyo, Japan) and were accurately diluted by keeping 0.2 M acid concentration.

A pH 4.5 acetate buffer solution was prepared by dissolving sodium hydroxide in 2 mol dm^{-3} acetic acid while measuring pH of the solution. The mobile phase was 46 or 48 %w/w aqueous acetonitrile solution containing 2 x 10^{-3} or 3 x 10^{-3} mol kg^{-1} of tetrabutylammonium bromide (TBA), 5 x 10^{-3} mol kg^{-1} of sodium acetate, and 10^{-4} mol kg^{-1} of ethylenediamine-tetraacetic acid (EDTA). EDTA acts as a masking agent for the metal contaminant from the HPLC apparatus. Distilled, deionized water was purified with a Millipore Milli-Q system.

Apparatus

The chromatographic system consisted of a Shimadzu LC6A pump, a Rheodyne 7125 injector with a 100-mm^3 sample loop and a Shimadzu SPD-6AV variable-wavelength spectrophotometric detector with a 10 mm flow-through cell. A Cosmosil 5TMS column (trimethyl-bonded phase column, 4.6 mm x 250 mm, Nacali Tesque, Kyoto, Japan) was used.

For measurement of the absorption spectra of the eluted chelates, a Shimadzu SPD-M10AVP photodiode-array detector was used, together with its associated software package. Sample weighing was performed with a Zartorius L420S electronic balance (readability: 1 mg).

Recommended Procedure

A sample solution (1-g portion) was accurately weighed into a 30-cm^3 screw capped Teflon bottle on an electronic balance. To the solution, 1 cm^3 of 2 x 10^{-3} M 5-Br-PADAP solution and 2 cm^3 of pH 4.5 acetate buffer solution were added. The whole mixture was diluted to 10 g with water on the balance and the weight of the resultant solution (W) was accurately weighed. The whole solution was tightly closed and heated on a boiling-water bath for 30 min and then cooled. Before and after heating, the weight of the solution (W) did not vary. The test solution, thus obtained, was injected with a 100-mm^3 loop injector onto the column. The rate of the mobile phase was 1.0 cm^3 min^{-1}. Chromatograms were obtained by measuring the absorbance at 595 nm.

The peak height (in mV and absorbance unit) of the chelates detected on the chromatograms was normalized, with dilution factor (W/10) calculated from the accurate weigh (W) of the test solution, i.e. (mV or absorbance unit) x W/10. The normalized values were used for determining the concentration of the metal ions.

Purification of Enzyme

Bromoperoxidase from *Corallina pilulifera* was purified from the crude extract with ammonium sulfate fractionation and the subsequent column chromatography, using a DEAE-Sepharose CL, a Sepharose 6B, and a Cellulofine GC-700m according to a previous report.[14] Bromoperoxidase, from *Pseudomonas putida* was also purified by the method previously reported.[11] Both solutions containing enzyme, were dialyzed in a cellulose-tubing for 2 days against several changes of water; the dialysis-inside solutions were used as samples to be analyzed and the outside solution containing no enzyme, was used as a blank.

Preparation of Enzyme Sample Solution

4-cm^3 of the inside solutions containing an enzyme and 4-cm^3 of the outside solution (as dialysis blank) obtained through the dialysis, were respectively heated with 4 cm^3 of concentrated nitric acid in a Teflon bottle on a hot-plate, nearly to dryness and then, each residue, with one drop of concentrated nitric acid, was diluted with water to 5-g on the electronic balance with accurate weighing. One-g portion of each solution, which was accurately weighed, was analyzed by the recommended procedure.

RESULTS AND DISCUSSION

Derivatization of Metal Ions with 5-Br-PADAP

The complexation of vanadium(V), cobalt(II), nickel(II), and iron(III) with 5-Br-PADAP was quantitative at pH 4.5 with acetate buffer solution.[15] The cobalt(II) chelate is easily oxidised to the cobalt(III) chelate which gives a net charge of +1 on the chelate. The test solution should be heated on a boiling-water bath to reduce the time of the derivatization since the color development of vanadium(V) and nickel(II) chelates with 5-Br-PADAP is slow; the heating time was fixed at 30 min.

Separation and Detection of 5-Br-PADAP Chelates

Among vanadium(V), cobalt(II), nickel(II), iron(III), cadmium(II), copper(II), zinc(II), manganese(II), and aluminum(III), four chelates, vanadium(V), cobalt(II) (as cobalt(III) chelate), nickel(II), and iron(III) (as iron(II) chelate as described below) chelates, gave well resolve peaks using an aqueous acetonitrile mobile phase independent of the addition of TBA. Here it was found that, even though the iron(III) chelate was injected onto the column, an eluted-iron chelate was the iron(II) chelate although it is not clear why iron(III) is reduced to iron(II). Because the spectrum of the eluted iron chelate, observed using a photo diode-array detector, agreed with that of iron(II) chelate, which has two maxima at 558 and 748 nm, measured with a conventional spectrophotometer; while iron(III) chelate has one maximum at 595 nm. The chelates of metal ions, such as copper(II) and zinc(II), decomposed on the column during elution and gave no peaks on the chromatogram; the chelates such as cadmium(II), manganese(II), and aluminum(III) chelates do not form at given condition, pH 4.5. As a result, these foreign metal ions did not interfere with the detection of vanadium(V), cobalt(II), nickel(II), and iron(III).

When TBA was absent in an acetonitrile-water mobile phase, the chelates eluted in the order of vanadium(V), nickel(II), iron(II), and cobalt(III) chelates and especially cationic cobalt(III) chelate, gave rise to slow elution. Since it took about 60 min to elute the cobalt(III) chelate, the chelate gave a broadened peak and poor detection. On the other hand, as TBA was added to the mobile phase, TBA constrained the cobalt(III) chelate to be eluted before the nickel(II) chelate and ensured highly sensitive for cobalt.[13] In the present method, the

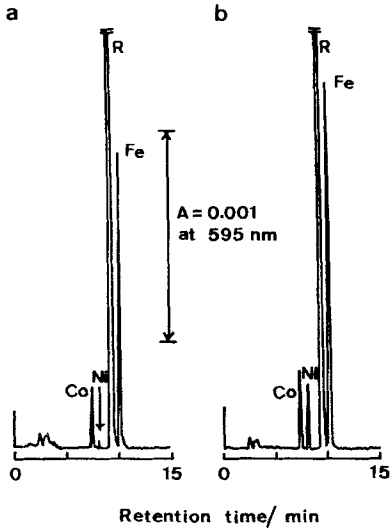

Figure 1. Analysis of bromoperoxidas from *Pseudomonas putida*. Mobile phase: 48 %w/w aqueous acetonitrile containing $3x10^{-3}$ mol kg^{-1} TBA;a: blank (outside solution); b: sample (inside solution containing enzyme);R: free 5-Br-PADAP.

cobalt(III) chelate could be eluted within 8 min and it took only 12 min per one run. TBA is adsorbed to the reversed phase(RP) stationary support based on the ion exchange and hydrophobic interaction mechanisms; from those results, the ion exchange adsorption of the cationic cobalt(III) chelate is prevented by preferential ion exchange of TBA cation with silanol proton and the chelate is excluded from the polar stationary phase, the surface of which is mostly covered by TBA cation.[16] Since the retention time of the cobalt(III) chelates decreased with increasing the concentration of TBA[13] and TAB was dynamically retained onto the RP stationary phase,[16] the concentration of TBA in the mobile phase should be kept at constant to preserve the uniform retention of TAB on the stationary support.

The retention time of all chelates also decreased with increasing acetonitrile concentration. The eluting conditions were optimized in view of the compromise between peak resolution and total elution time. The separation of each chelate from other chelates was optimized by varying the concentration of acetonitrile and the retention time of the cobalt(III) chelate was adjusted by changing TBA concentration.

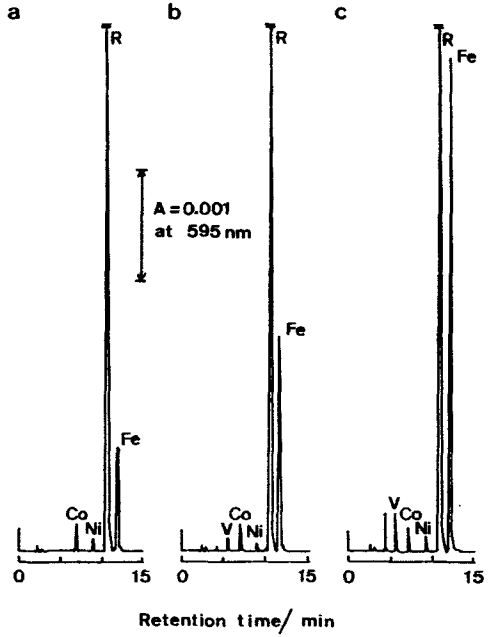

Figure 2. Analysis of bromoperoxidases from *Corallina pilulifera*. Mobile phase: 46 %w/w aqueous acetonitrile containing $3x10^{-3}$ mol kg^{-1} TBA;a: blank (outside solution); b: sample 1 (inside solution 1 containing enzyme);c: sample 2 (inside solution 2 containing enzyme); R: free 5-Br-PADAP.

The concentrations of acetonitrile and TBA in a mobile phase were chosen in the range from 46 to 48 %w/w acetonitrile and the range from 2 x 10^{-3} to 3 x 10^{-3} mol kg^{-1} TBA, respectively. Addition of TBA to the mobile phase should serve to establish simultaneous and rapid HPLC determination for these chelates.

When a 100-mm^3 aliquot of a test solution was injected onto the column with 46 %w/w or 48 %w/w acetonitrile mobile phase containing 3 x 10^{-3} mol kg^{-1} of TBA, the peak height calibration curves (0.005 absorbance unit full scale) of vanadium(V), cobalt(II), nickel(II), and iron(III) linear up to 300 pg of vanadium(V), 160 pg of cobalt(II), 2400 pg of nickel(II), and 1500 pg of iron(III) in 100 mm^3-injection, respectively. When 80 pg of vanadium(V), 40 pg of cobalt(II), 240 pg of nickel(II), and 1000 pg of iron(III) were injected and determined, the relative standard deviations for three determinations of vanadium(V), cobalt(II), nickel(II), and iron(III) were 2.8 %, 1.1 %, 4.3 % and 0.8 %, respectively.

Table 1

Determination of Vanadium and Iron in *Corallina pilulifera*

Sample No.		Concentration of Metal Ions, ng/cm^3-Dialyzed Solution (mol/mol/enzyme) This Method	ICP-AES*	ICP-MS**
1	V	4.5 ± 0.5 (0.42)	3.0 (0.28)	3.1 (0.29)
	Fe	97 ± 17 (8.3)	101 (8.7)	64 (5.5)
2	V	12.0 ± 0.8 (0.53)	11.0 (0.49)	13.0 (0.58)
	Fe	320 ± 4 (12.9)	266 (10.8)	198 (8.0)

* inductively coupled plasma atomic emission spectrometry.
** inductively coupled plasma mass spectrometry.

The detection limits, defined as the concentration corresponding to 3 times the standard deviation of the reagent blank, were 2.2 pg of vanadium(V), 0.72 pg of cobalt(II), 16.5 pg of nickel(II), and 98.8 pg of iron(III) in a 100-mm^3 injection, respectively, when elution conditions were fixed at 46 %w/w acetonitrile and 2 x1 0^{-3} mol kg^{-1} TBA.

Analysis of Enzyme

The present method was applied to the determination of vanadium, cobalt, nickel, and iron in the bromoperoxidases from *Pseudomonas putida* and *Corallina pilulifera*. A 1-g portion of the solution treated with nitric acid was analyzed by the recommended method.

Figure 1 shows typical chromatograms obtained in the analysis of *Pseudomonas putida* by the proposed method. As shown in Fig. 1-a, even if no metal ion was added to the test solution as a blank, the peaks corresponding to cobalt(III), nickel(II), and iron(II) chelates were caused on the chromatogram. This is mainly attributed to the contaminant from the reagents

used. The chromatogram was used as the blank chromatogram. In 1 cm^3 of the dialyzed solution, 1.3 ng of cobalt, 54.6 ng of nickel and 6.7 ng of iron were found, respectively, and vanadium was not detected. From these results, we would reveal a novel class of bacterial bromoperoxidase.[11]

Figure 2 was obtained from analyses of the blank solution(a) and of the bromoperoxidases (b: sample 1, c: sample 2) from *Corallina pilulifera.* Table 1 gives the results of determination of vanadium and iron in the bromoperoxidases from *Corallina pilulifera.* For comparison, the data obtained with inductively coupled plasma (ICP) atomic emission spectrometry and ICP mass spectrometry (MS) are also listed. Vanadium and iron in the enzymes can be successfully determined by the proposed method.

The proposed method is one of the most sensitive methods for vanadium and cobalt; the detection limits of both metal ions are comparable with or lower than those reported for ICP-MS which were 23 - 30 pg cm^{-3} for vanadium and 0.49 - 10 pg cm^{-3} for cobalt , respectively.[17,18] The method here provides a selective and sensitive determination of vanadium, cobalt, nickel, and iron, and is applicable to the analysis of various enzymes.

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