Journal of Chromatography, 303 (1984) 131–136
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 16,943

SEPARATION OF METALLOTHIONEIN INTO ISOFORMS BY COLUMN SWITCHING ON GEL PERMEATION AND ION-EXCHANGE COLUMNS WITH HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY–ATOMIC-ABSORPTION SPECTROPHOTOMETRY

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(Received March 19th, 1984)

SUMMARY

Metallothionein in tissue supernatant was separated into isoforms by on-line switching from gel filtration to ion-exchange columns and the metals bound to the isoproteins were directly determined by using a flame atomic-absorption spectrophotometer as a specific detector for metals. Conditions for elution of the gel filtration column were established and Tris–HCl buffer solutions in low concentration were shown to be applicable to the column-switching experiment. The effects of pH, buffer and salt concentrations on the elution of metallothionein are discussed.

INTRODUCTION

Metallothionein, a low-molecular-weight protein rich in cysteinyl residues and heavy metals has been shown to be a ubiquitous metal binding protein present and/or inducible among various living bodies[1]. However, biological activities that can be used as bioassays for the protein are not known. Although a sensitive radioimmunoassay method has been developed[2–4], it can be used only for known metallothionein samples. Hence, unknown metallothioneins or metallothionein-like proteins have to be characterized after separation by appropriate methods.

The conventional method for the separation of metallothionein consists in a combination of gel filtration and ion-exchange columns and the protein is quantified by the metals bound to the protein. We have previously reported an analytical method for metallothionein that detects the bound metals after separation by a combination of high-performance liquid chromatography (HPLC) on a gel permeation column and atomic-absorption spectrophotometry (AAS)[5,6]. Several advantages of

0021-9673/84/$03.00

this method over the conventional method were pointed out, the most important being the separation of metallothionein into isoproteins by elution with alkaline buffer on an SW column.

Although metallothionein can be separated into the two isometallothioneins on an SW column by HPLC–AAS, its separation is not as satisfactory as separation on an ion-exchange column[7]. Therefore, it is desirable to separate metallothionein completely into isoforms by a combination of gel filtration and ion-exchange columns with HPLC–AAS in order to obtain precise information on the isoforms, including structural changes induced by metal exchange and/or disulphide bond formation.

The purpose of this study was to develop a more elaborate method for the separation of isoforms of metallothionein by an on-line combination of gel filtration and ion-exchange columns using an HPLC–AAS method. For the separation and analysis of the metallothionein fraction on a gel filtration column, the fraction was introduced into an ion-exchange column by switching columns through a four-way valve and the ion-exchange column was eluted under conditions suitable for separation of the isoforms[7]. Elution conditions for the gel filtration column were examined with a view to direct introduction of the eluate into an ion-exchange column, and several requirements for this method are considered.

EXPERIMENTAL

Chemicals

Tris–HCl buffer solutions were prepared by mixing Trizma base and Trizma hydrochloride as indicated by the manufacturer (Sigma, St. Louis, MO, U.S.A.) and sodium azide was purchased from Wako (Osaka, Japan). Doubly distilled water was used for all experiments.

Metallothionein was prepared as follows. Cadmium chloride was injected subcutaneously into female Wistar rats (8 weeks old; mean body weight 115 g) at a dose (as cadmium) of 3.0 mg/kg body weight four times a week for 4 weeks and the animals were killed 12 weeks after the last injection. The livers were homogenized in 4 vol. of 0.1 *M* Tris–HCl buffer (pH 7.4, 0.25 *M* glucose) using a Polytron homogenizer in an atmosphere of nitrogen with ice–water cooling and the homogenate was centrifuged at 170,000 *g* for 60 min at 2°C.

Apparatus

HPLC for gel permeation chromatography was carried out using an HLC 803A pump (Toyo Soda, Tokyo, Japan) for solvent delivery, with a column [TSKgel G 3000 SW (600 × 7.5 mm I.D.) equipped with a TSK SW guard column (75 × 7.5 mm I.D.); Toyo Soda], a dual-wavelength ultraviolet detector (Model 152, Altex, Berkeley, CA, U.S.A.) and an atomic-absorption spectrophotometer with an air–acetylene flame (Hitachi 170-50A). Column-switching experiments were performed on an HPLC Series instrument 340 (Beckman, Berkeley, CA, U.S.A.), with an SW column (600 × 7.5 mm I.D., equipped with a guard column) and an ion-exchange column (TSKgel DEAE-3SW, 150 × 6.0 mm I.D.; Toyo Soda), four-way valves (Gasukuro Kogyo, Tokyo, Japan), a conductivity meter (CD-35MII, M & S Instruments, Tokyo, Japan), a dual-wavelength ultraviolet detector and an atomic-absorption spectrophotometer with an air–acetylene flame as shown in Fig. 1.

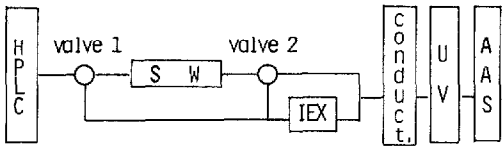

Fig. 1. Schematic diagram for column switching of SW and ion-exchange columns using the HPLC–AAS method. HPLC = HPLC Series 340 chromatograph (Beckman). SW = TSKgel G3000SW gel filtration column (600 × 7.5 mm I.D., with a guard column of 75 × 7.5 mm I.D.). IEX = TSKgel DEAE-3SW ion-exchange column (150 × 6 mm I.D.). Conduct. = conductivity meter (CD-35MII, M & S Instruments). UV = dual-wavelength ultraviolet detector (Model 152, Altex). AAS = Hitachi AA 170-50A flame atomic-absorption spectrophotometer. Valves = four way-valves (Gasukuro Kogyo).

RESULTS AND DISCUSSION

Fig. 1 is a schematic diagram of the column-switching experiment. For delivery of three different solvents, three HPLC pumps were used; the first pump was used for continuous delivery of buffer solution to the SW column, and a linear concentration gradient of buffer solution for elution of the ion-exchange column was prepared by the second and third pumps. A sample solution was introduced into the SW column through valve 1 and the eluate was monitored consecutively by a conductivity meter, ultraviolet detector and atomic-absorption spectrophotometer. Peak positions of appropriate fractions such as the metallothionein fraction on the SW column were calculated by subtracting the time equivalent to the volume of the connecting tubes from the retention times and the specified fraction on the SW column was selectively introduced into the ion-exchange column through valve 2. The ion-exchange column was eluted by delivering solvent through valve 1 and the eluate was continuously monitored consecutively by the three detectors. After regenerating the ion-exchange column and equilibrating with the starting buffer solution, the second fraction on the SW column can be introduced into the ion-exchange column by switching the two valves and elution of the ion-exchange column can be repeated.

For the retention of samples transferred from the gel filtration column to the anion-exchange column, the buffer solution for the gel filtration column should have a low concentration and a reasonably high pH. As the pH-dependent separation of the two isometallothioneins has been achieved on an SW column[5,6], the effects of the pH and concentration of the buffer solution on the separation of the two isometallothioneins were examined in order to determine the optimal conditions for the column-switching experiment. The two isometallothioneins were separated better at higher pH at both Tris concentrations examined (Fig. 2). However, the separation was less affected at a lower buffer concentration. The retention times of the two isometallothioneins were shorter at higher pH values at both buffer concentrations, and were shorter at lower buffer concentrations at the same pH. Elution using 10 m*M* Tris–HCl buffer (pH 8.6) gave the shortest retention time for metallothionein in Fig. 2 and the two isometallothioneins were separated reasonably well under these conditions.

Although elution at higher pH was shown to give a good separation of the two isometallothioneins, it is recommended not to elute an SW column at a pH higher than 8.0 in order to prevent destruction of the column materials. A lower buffer concentration was also shown to give a better separation than a high buffer concen-

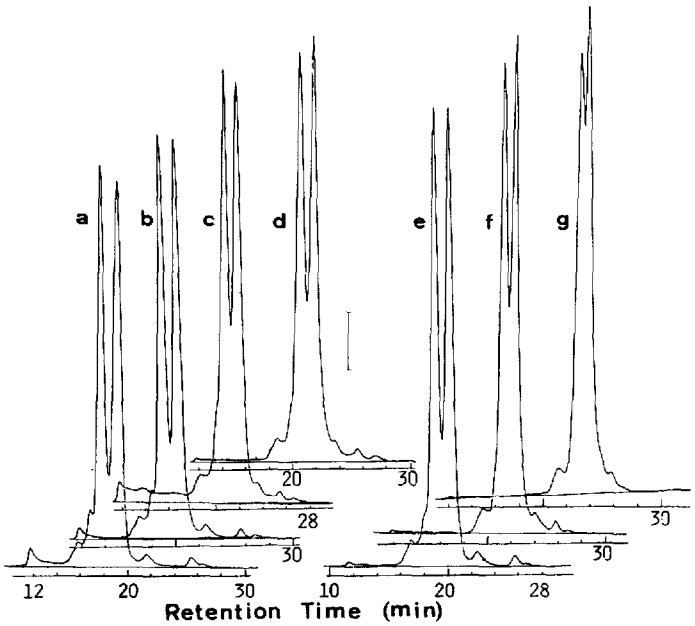

Fig. 2. Elution profiles of liver supernatant on the SW column under various elution conditions using the HPLC–AAS method. A 0.1-ml portion of liver supernatant prepared from repeatedly Cd-injected rats was applied to the SW column, which was eluted with Tris–HCl buffer solutions (containing 0.1% of sodium azide) at various pHs and concentrations, using the HPLC–AAS method. The concentration of the elution buffers for profiles a (pH 8.6), b (pH 8.0), c (pH 7.4) and d (pH 7.2) was fixed at 10 m*M* and that for profiles e (pH 8.6), f (pH 8.0) and g (pH 7.2) was fixed at 50 m*M*. Each buffer solution was pumped at a flow-rate of 1.0 ml/min. The concentration of cadmium in the eluate was continuously recorded by connecting the outlet of the column to the nebulizer tube of the flame atomic-absorption spectrophotometer (Hitachi AA 170-50A). The vertical bar indicates the AAS detection level (0.1 μg/ml of Cd).

tration at the same pH, but it is not known why this occurs. It is also not clear why metallothionein is eluted faster at lower buffer concentrations. Sodium azide, added to buffer solutions at a concentration of 0.1% as a preservative for the column, was also shown to affect the elution and separation of the two isometallothioneins; metallothionein was eluted almost at the void volume of the column when the column was eluted without sodium azide using 10 m*M* Tris–HCl buffer solutions, especially at higher pH (data not shown). The effect of sodium azide on the elution was not obvious when using 50 m*M* Tris–HCl buffer solutions. Therefore, the effect of sodium azide is probably similar to or the same as that of buffer concentration.

Elution conditions for the column-switching experiment were examined as shown in Table I. In this experiment, the SW column was eluted under eight different conditions and the ion-exchange column was eluted under the same conditions for the eight elutions as employed in a previous experiment[7]. The elution conditions were evaluated from the elution profiles on the ion-exchange column. A low buffer concentration (10 m*M*) was applicable at pH values between 7.2 and 8.6, whereas a high buffer concentration (50 m*M*) was applicable only at pH 8.6. At the high buffer concentration metallothionein, especially metallothionein I, was not retained on the ion-exchange column during transfer from the SW column and was eluted before or

TABLE I

ELUTION CONDITIONS FOR ON-LINE SWITCHING FROM AN SW TO AN ION-EXCHANGE COLUMN USING THE HPLC–AAS METHOD

Eight elution conditions for the SW column were examined and evaluated according to the elution profiles on the ion-exchange column. A 0.1-ml portion of rat liver supernatant was applied to the SW column and the column was eluted with Tris–HCl buffer solutions (containing 0.1% of sodium azide) at a flow-rate of 1.0 ml/min. The metallothionein fraction was directly applied to the ion-exchange column by column switching. The ion-exchange column was eluted with a linear concentration gradient of Tris–HCl buffer (pH 7.2) by mixing starting buffer (2 m*M*) and limiting buffer (50 m*M*) up to a 50 m*M* concentration during 20 min. The elution conditions for the SW column were evaluated from elution profiles on the ion-exchange column.

Buffer concentration (mM)	*pH*			
	7.2	*7.4*	*8.0*	*8.6*
10	Good	Good	Good	Good
50	Bad	Bad	Poor	Good

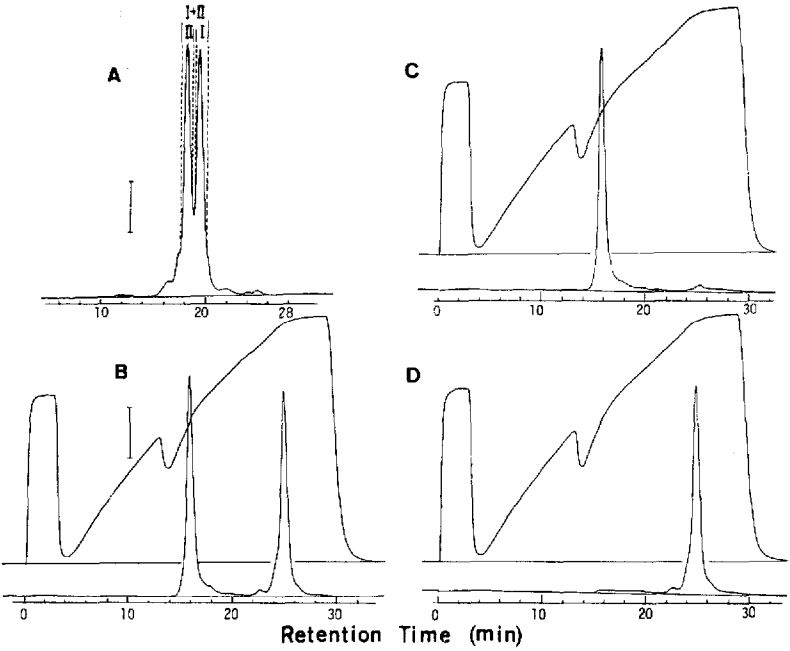

Fig. 3. Elution profiles of metallothionein (A) on the SW column and (B–D) on the ion-exchange column after column switching from the SW to the ion-exchange column using the HPLC–AAS method. A 0.1-ml portion of the liver supernatant was applied to the SW column, which was eluted with 10 m*M* Tris–HCl buffer solution (pH 8.0, containing 0.1% sodium azide) at a flow-rate of 1.0 ml/min. The eluate was directly introduced into the AAS nebulizer tube and cadmium in the eluate was continuously monitored (profile A). For the column-switching experiment, the whole (I + II) of the metallothionein fraction on the SW column shown in profile A was introduced into the ion-exchange column (TSKgel DEAE-3SW), which was eluted with a linear concentration gradient of Tris–HCl buffer solution (pH 7.2) from 2 m*M* (starting buffer concentration) to 50 m*M* (limiting buffer concentration) at a flow-rate of 1.0 ml/min. The eluate from the ion-exchange column was also continuously monitored. Profiles B, C and D were obtained by introducing the metallothionein fractions I + II, I and II of the profile A, respectively.

just after the application of a gradient buffer solution for the ion-exchange column.

Typical elution profiles obtained in the column-switching experiment are shown in Fig. 3. The peak width on the SW column (Fig. 3A) was broad compared with that in Fig. 2b. This is primarily due to the complex and long tubing, as indicated in Fig. 1, compared with the simple HPLC–AAS method applicable to Fig. 2. Introduction of the metallothionein fraction (fraction I + II in Fig. 3A) from the SW column on to the ion-exchange column by switching columns and elution of the ion-exchange column with a concentration gradient gave the elution profile shown in Fig. 3B. The stepwise introduction of the metallothionein fraction (fraction II and then I) from the SW column on to the ion-exchange column and repeated elution of the two fractions gave two clearly isolated isometallothioneins, as shown in Fig. 3D and C, respectively. Thus, the on-line connection of the two columns with different separation principles was efficient for the separation and characterization of proteins in a complex mixture. The HPLC–AAS method was found to be applicable also to the column-switching experiment.

ACKNOWLEDGEMENT

K.T.S. and H.S. thank Dr. K. Kubota (NIES) for his continuous encouragement.

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