ISOLATION AND PURIFICATION OF CADMIUM BINDING PROTEINS FROM RAT LIVER

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Summary A simple procedure for the isolation and pruification of metallothionein from rat liver is described. This method involves only four steps and is especially useful for large scale isolation of this protein. The final isolated preparation was homogeneous both in Sephadex gel filteration and in polyacrylamide gel electrophoresis. Isoelectric focussing shows the presence of two cadmium binding proteins with isoelectric points of 4.2 and 4.7. Metallothionein is isolated from dog liver using this method.

Introduction Metallothionein, a protein with high affinity for cadmium was first isolated and characterised by Margoshes and Vallee (1). This cytoplasmic protein has a low molecular weight of about 11,000 daltons and is not present in liver normally in animals. But a number of workers have shown that it is actively synthesized in the liver of various species namely rat (2), rabbit (3), mice (4), and chicken (5) on injection of small amounts of cadmium salts. The exact role of this protein on the absorption, transport and storage of cadmium is not known (6). Recent reports suggest that this protein may be involved in the metabolism of other metals (7, 8) like copper, zinc and mercury.

Most of the previous methods (3, 9-11) used for the isolation of metallothionein were laborious involving several steps and had poor recovery. Shaikh and Lucis (12) have developed a method using ion exchange resins for isolation of metallothionein for analytical purposes. The present report describes a simple isolation procedure for metallothionein. It can be used for the isolation of this protein in small amounts to study its metabolism or the purification in a preparative scale to study its biological properties.

Materials and Method Female Sprague-Dawley rats weighing 150-220 grams

were injected subcutaneously with a dose of 0.25 mg/Kg of cadmium chloride for six days. The rats were injected with one μCi of carrier free radioactive $^{109}CdCl_2$ 24 hours before sacrificing. Livers were homogenized (20% weight/volume) in 0.25 M sucrose, 0.1 M phosphate buffer, pH 7.4. The homogenate was centrifuged at 37,000 g for 15 minutes at 4° C to remove nuclear and mitochondrial fractions. The post-mitochondrial supernatant was heated in a water bath to 70° C and maintained at that temperature for one minute. The sample was chilled immediately, then centrifuged to obtain the heated supernatant fraction which was subjected to ammonium sulfate fractionation. In the initial step, ammonium sulfate was added slowly to the heated supernatant with stirring to a concentration of 25% weight/volume in the cold and was centrifuged after 20 minutes. The pellet was discarded and more ammonium sulfate was added to the supernatant to saturation (60 mg/ml). The salting-out was completed in the cold within 30 minutes and the solution was centrifuged. The pellet was dissolved in a minimum volume of 0.1 M phosphate buffer, pH 7.4 and dialysed against distilled water to remove excess of ammonium sulfate. The solubilized material was fractionated on a calibrated Sephadex G-75 column (2.5 x 90 cm) equilibrated with 0.01 M ammonium formate, pH 7.4, buffer. The elution from the column was monitored at 280 mμ and 254 mμ by LKB Uvicord III and 1 ml fractions were counted for ^{109}Cd in a Packard scintillation spectrometer equipped with a sodium iodide detector with a counting efficiency of 40%. ^{109}Cd containing fractions emerged between V_e/V_o of 1.6 to 2 from Sephadex G-75 columns, had high absorbance at 254 mμ, were combined and concentrated in a Vir Tris freeze drier.

Cadmium was determined by atomic absorption method (13) and protein was estimated by the method of Lowry et al (14). Polyacrylamide gel electrophoresis was carried out by the method of Davis (15) and isoelectric focussing in sucrose gradient was done in LKB 8100 ampholine equipment by the method of Flatmark and Vesterberg (16) using 3-6 ampholines.

Results A typical experiment showing the different steps of purification

Table 1. Purification of Cadmium Binding Protein and Recovery of Cadmium

Purification Step*	Protein mg	Cadmium µg	% Recovery of Cd	µgCd/mg protein
Homogenate		11950	100.0	
Supernatant 37,000 g	16600	10330	86.5	0.62
Heated Supernatant	4600	9400	78.7	2.04
Ammonium sulfate (25%-saturation)	1560	7740	64.8	4.96
Sephadex G-75 V_e/V_o 1.6 - 2	270	7430	62.2	27.61

*For details see Materials and Method.

and recovery of cadmium and protein is shown in Table 1. More than 70% of the proteins were removed from the supernatant with 90% cadmium recovery achieved in the heating step. The percentage recovery of cadmium in each step was comparable from one experiment to another and the final recovery was between 73% to 61%.

The only main loss of cadmium was during the salting-out step, if the proper cooling conditions and concentration of ammonium sulfate were not maintained. The Sephadex G-75 filtrate gave only one main protein peak containing cadmium which emerged almost in the same position as ribonuclease (V_e/V_o 1.6-2). This peak could account for 98% of the cadmium applied on the column.

The final purified sample when rechromatographed on Sephadex G-75 column gave only one single cadmium peak and a corresponding 254 mµ absorption peak with a molecular weight of about 11,000 (Fig. 1).

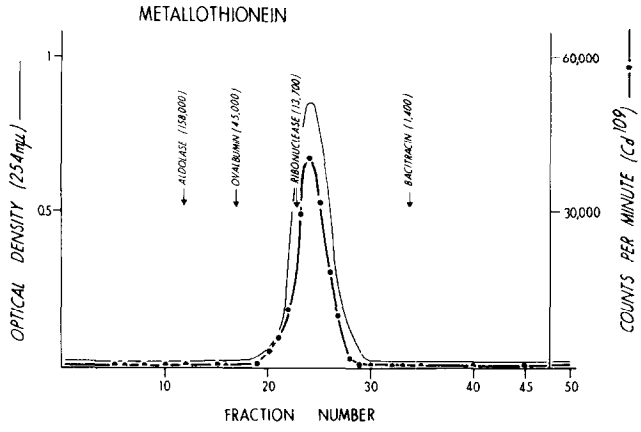

Fig. 1. Sephadex G-75 filteration of the final purified metallothionein labelled with ^{109}Cd from rat liver and standard samples of aldolase, ovalbumin, ribonuclease and bacitracin (Pharmacia Fine Chemicals).

Fig. 2. Polyacrylamide gel electrophoretic separation by the method of Davis (15). A. Liver supernatant. B. Heated super. C. Sephadex G-75 fraction (30 μg protein). D. Same as C (70 μg).

The gel electrophoresis of the samples from different steps during purification and the final purified metallothionein are shown in Fig. 2. The electrophoretic pattern of the final purified sample gave one prominent and a minor band after protein staining with Commassiee blue.

The purity of the isolated proteins was also established by isoelectric focussing in sucrose density gradient using ampholines pH 3 to 6. This gave two protein peaks and corresponding cadmium radioactive peaks (Fig. 3) with isoelectric points 4.2 and 4.7. This shows the presence of only two cadmium binding proteins in the final purified sample and both of them may represent cadmium binding proteins reported by Nordberg et al (11) and Shaikh and Lucis (12).

Discussion A simple method for isolation and purification of cadmium binding proteins from rat liver is described. The various steps used in this method are based on the different properties of this protein namely its subcellular localization, heat stability and low molecular weight.

Webb (17) has reported that the metallothioneins from rat liver are heat stable and they share similar properties with iron storage protein ferritin in heat treatment. A heating step at 70° C for one minute is introduced in the present isolation method on the supernatant fraction and this

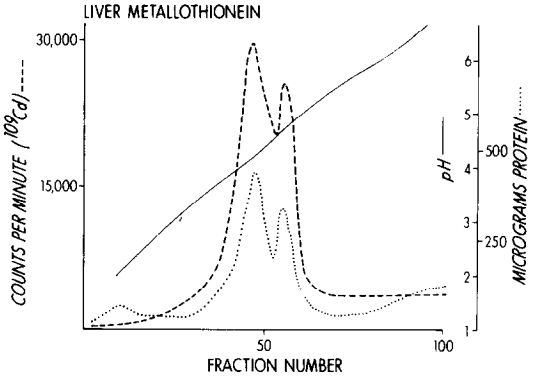

Fig. 3. Isoelectric focussing of the final purified sample in sucrose gradient with ampholines pH 3 to 6 by the method of Flatmark and Vesterberg (16).

could remove more than 80% of total protein from the supernatant fraction with seven-fold purification of cadmium binding proteins (Table 1). This step also destroys most of the proteases present in this fraction and it may increase the total recovery of the cadmium binding proteins during the isolation. However, Webb (17) has reported that metallothioneins are not readily hydrolyzed by proteases. The heat treatment of the liver supernatant at 70^0 C for one minute did not denature metallothionein because no cadmium was lost from the 10,000 molecular weight protein during this step. The identical isoelectric points of the metallothioneins in isoelectric focussing before and after heating also support the heat stable property of metallothionein.

A fraction of the high molecular weight proteins in the heated supernatant was removed by 25% ammonium sulfate fractionation. The cadmium binding proteins with a molecular weight of 10,000 are salted out only between ammonium sulfate concentration of 25% and saturation. The concentration of the sample volume for the Sephadex fractionation was also achieved by this step. The main purification of the protein was achieved in the Sephadex G-75 fractionation. Ammonium formate was used as an eluant buffer in this step to get a salt-free preparation after freeze drying.

In large scale isolation procedures of metallothionein, to reduce the total volume of the starting material, a fifty percent homogenate of the tissue can be used instead of a twenty percent homogenate as mentioned in this report. However, the final recovery of cadmium is lower when a tissue homogenate more concentrated than twenty percent is used as the starting material.

The polyacrylamide gel electrophoresis and isoelectric focussing show only the presence of cadmium binding proteins in the final purified sample. There was only one major protein band and almost all the cadmium in the purified fraction was associated with this band. It is possible to isolate this fraction into two main protein components using isoelectric focussing. However, both these components could bind with cadmium and represent metallo-

thioneins confirming previous reports (10, 12).

This simple method can be employed to isolate pure cadmium binding proteins in large quantities using suitable Sephadex columns and it can be adapted to different tissues in various species with little modification. We have used the same method to isolate metallothionein from dog liver in large quantities to study its physiological properties (18). The biological role of this protein in the metabolism of heavy metals are under investigation in a number of laboratories and it is speculated that it acts as a protective agent against the toxic effects of heavy metals. Our preliminary results (6) suggest that cadmium administered in the form of metallothionein is accumulated in the kidney unlike cadmium chloride, which is mainly stored in the liver. This simple procedure for the isolation of pure cadmium binding proteins may be useful in studying the metabolic fate of this protein and its influence on the normal metabolism of cadmium, mercury and other heavy metals.

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