Note

A Study on the Cd-hem Method for the Simple Determination of Metallothionein in Biological Samples[†]

Itaru SATO, Naonori MATSUSAKA, Shuji TSUDA and Toichi HIRATA

Faculty of Agriculture, Iwate University
3-18-8, Ueda, Morioka-shi 020-8550, Japan

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Cd-hem method is the most simple and easy method for the determination of metallothionein (Mt). In this method, Mt-unbound Cd is removed by a treatment with rat hemoglobin (Hb) and heating at 100 °C. This step is usually repeated 3 times. The authors have carried out several experiments on the Cd-removal process in the Cd-hem method.

Bovine and swine Hbs have a high affinity for Cd and are satisfactorily available for the Cd-hem method instead of the rat Hb. Although Mt is not lost by the treatment with Hb and heating at 100 °C, only a single treatment is recommended because it is enough for the removal of Mt-unbound Cd. For the accurate determination of Mt, the value of blank which is determined without biological samples should be subtracted from that of biological samples because the Mt-unbound Cd cannot be removed completely by this treatment even though the treatment is repeated 3 times.

Key Words: metallothionein, simple determination, cadmium-hem method

1. Introduction

Metallothionein (Mt), which was first isolated from equine renal cortex in 1957¹⁾ is a cysteine-rich low molecular weight protein²⁾. It has several biological functions such as detoxication of heavy metals^{3), 4)}, control of zinc homeostasis^{5), 6)}, and protection against radiation injury⁷⁾. Although many methods such as gel permeation chromatography⁸⁾, Hg-TCA method⁹⁾, high performance liquid chromatography¹⁰⁾, polarographic method¹¹⁾, and radioimmunoassay¹²⁾ have been developed for the determination of Mt in biological samples, the most simple method is the

Cd-hem method reported by Onosaka et al.¹³⁾. This method consists of 3 steps. The first step is saturation of the metal binding sites of Mt with Cd. The second step is removal of the Mt-unbound Cd by a treatment with rat hemoglobin (Hb). This step is repeated 3 times. The third step is determination of the Cd concentration in the supernatant fraction. Although this is a simple and easy method for the determination of Mt, we have the following questions on the second step of the Cd-hem method.

(A) Can Hb solution prepared from the other animal species not be used instead of the rat Hb?

(B) Must the second step be repeated 3 times?

(C) Are there no any loss of Mt during the repeated treatment with Hb?

The present study was carried out to solve these

[†]メタロチオネイン簡易定量法Cd-hem法に関する一考察。佐藤 至, 松坂尚典, 津田修治, 平田統一: 岩手大学農学部, 020-8550 盛岡市上田 3-18-8。

questions.

2. Experimental Methods

2.1 Preparation of hemolysates

Bovine (Holstein), swine (hybrid) and rat (Wistar) hemolysates were prepared as follows¹⁴⁾. Ten milliliters of the blood was mixed with 20 ml of 1.15% KCl, and centrifuged at 3 000 *g* for 5 min. The precipitate was suspended in 30 ml of 1.15% KCl, and centrifuged on the same conditions. This step was repeated twice. Red blood cells in the precipitate were lysed by adding 20 ml of 30 mM Tris-HCl buffer, pH 8.0. The hemolysate was centrifuged at 9 000 *g* for 10 min to remove the membrane fraction. The supernatant fraction rich in Hb was used for the Cd-hem method. Hb concentration in the hemolysates was determined with a blood analyzer (Microcell Counter F-800, Sysmex).

2.2 Comparison of the Cd binding ability of Hb among 3 mammalian species

One milliliter of 10 ppm Cd (CdCl_2) solution was added to 2.4 ml of the Tris buffer. This solution was mixed with 0, 40, 80, 120, 160 or 200 μl of the bovine, swine or rat hemolysate, incubated at room temperature for 5 min, and heated in boiling water for 1 min to coagulate the Hb. This step was repeated 3 times. These solutions were adjusted to a volume of 4.0 ml with the Tris buffer, and centrifuged at 1 000 *g* for 5 min to remove the Hb-bound Cd. Cd concentration in each supernatant fraction was determined by atomic absorption spectrophotometry.

2.3 Comparison of the Cd removal efficiency between a single and repeated treatments with Hb.

One milliliter of 10 ppm Cd (CdCl_2) solution, 2.4 ml of the Tris buffer, and 0, 120, 240, 360,

480, or 600 μl of the bovine, swine or rat hemolysate were mixed. Although the treatment with hemolysate was performed only once, the other procedures were the same as described above. Cd concentrations thus obtained were compared with those obtained in the above experiment.

2.4 Estimation of the loss of Mt during the treatment with Hb.

An adult male mouse of the ICR strain was injected subcutaneously with ZnSO_4 (0.1 mmol/kg) to induce the Mt synthesis. Another mouse received no treatment. Twenty-four hours later, the mice were killed under ether anesthesia to collect the liver. The liver was homogenized in 4 volumes of 250 mM sucrose, and centrifuged at 18 000 *g* for 20 min at 4°C. Mt content in the supernatant fraction was determined by the Cd-hem method with various numbers of the treatment with Hb.

3. Results and Discussion

3.1 Comparison of the Cd binding ability of Hb among 3 mammalian species

Figure 1 shows the Cd concentration in the supernatant fraction of the solutions treated with the bovine, swine or rat hemolysate under various conditions. Cd concentration in the supernatant fractions which were not treated with hemolysate was around 2.4 $\mu\text{g}/\text{ml}$, while that of the hemolysate-treated groups was less than 0.025 $\mu\text{g}/\text{ml}$ except a group of the single treatment with 0.12 ml of the bovine hemolysate. Hemolysates of the 3 mammalian species effectively removed Cd from the solutions, and more than 99% of the Cd removal efficiency was achieved by the treatment with 0.24 ml or more hemolysates.

Although there was no significant difference in the Cd removal efficiency between the bovine and rat hemolysates, the swine hemolysate appeared to

Fig. 1 Removal of Cd by the treatment with bovine, swine and rat hemolysates, and effects of volume of the hemolysate and number of the treatment on the Cd-removal efficiency.

Dotted column : Single treatment, hemolysate was added in one portion.

Striped column : Triple treatments, hemolysate was added in 3-divided dose.

be a little more effective than the others. Hb concentration in the bovine, swine, and rat hemolysates was 48, 74, and 62 mg/ml, respectively. However, the difference in the Cd removal efficiency may be due to the difference in the affinity of Hb for Cd, because a dose-dependent effect was not observed when the volume of hemolysate was increased above 0.24 ml.

Onosaka and Cheria¹⁴⁾ have mentioned that the rat Hb has a high affinity for Cd, but our result demonstrates that not only rat Hb but also bovine and swine Hbs have a high affinity for Cd. Therefore, bovine and swine hemolysates can be used instead of the rat hemolysate.

3.2 Comparison of the Cd removal efficiency between a single and repeated treatments with Hb.

The Cd removal efficiency of 0.24 ml hemolysate was significantly higher than that of 0.12 ml hemolysate. However, there was no further increase in the Cd removal efficiency when the volume of hemolysate was increased above 0.24 ml, and 0.008–0.015 µg-Cd/ml was left in the solutions even when the treatment with hemoly-

sate was repeated 3 times (Fig. 1).

Onosaka et al.¹³⁾ examined the effect of dilution of the hemolysate on the Cd removal efficiency, and found that the rat hemolysate could completely remove the free Cd up to 50 times dilution, which is equivalent to merely 0.012 ml of the original hemolysate. Although they did not determine the Hb concentration in the hemolysate, the concentration of their hemolysate must be similar to ours since we prepared the hemolysate according to the method of them. Nevertheless, our result demonstrates that the treatment with Hb cannot remove the free Cd completely.

Although Cd was removed more effectively by repeating the treatment with hemolysate 3 times, the difference in Cd concentration between the solutions treated once and those treated 3 times was only 0.005 µg/ml. Surplus Cd of 0.005 µg/ml causes an overestimate of 1.8 µg-Mt/g-tissue if 1 ml of 10% biological samples is assayed by this method. However, 0.008–0.015 µg-Cd/ml was left in the solutions even after the treatment with hemolysate was repeated 3 times. Therefore, the value of blank which is determined without biological samples should be subtracted from that of

Table 1 Effect of number of the treatment with Hb on the estimation of Mt ($\mu\text{g Mt/g tissue}$)

Treatment with Hb	Gross Mt	Blank	Net Mt
< Zn-treated mouse >			
Once (0.6ml $\times$ 1)	83.9 $\pm$ 0.4	4.6 $\pm$ 0.2	79.3 $\pm$ 0.4
Twice (0.3ml $\times$ 2)	83.3 $\pm$ 0.3	3.6 $\pm$ 0.2	79.7 $\pm$ 0.3
3 times (0.2ml $\times$ 3)	82.9 $\pm$ 0.5	3.0 $\pm$ 0.2	79.9 $\pm$ 0.5
< Untreated mouse >			
Once (0.6ml $\times$ 1)	11.2 $\pm$ 1.0	4.6 $\pm$ 0.2	6.6 $\pm$ 1.0
Twice (0.3ml $\times$ 2)	9.8 $\pm$ 0.5	3.6 $\pm$ 0.2	6.2 $\pm$ 0.5
3 times (0.2ml $\times$ 3)	9.5 $\pm$ 0.6	3.0 $\pm$ 0.2	6.5 $\pm$ 0.6

Mean $\pm$ SD for quadruplicate measurements.

biological samples to estimate the Mt content accurately.

3.3 Estimation of the loss of Mt during the treatment with Hb.

Table 1 shows hepatic Mt contents in mice. One mouse was given 0.1 mmol-Zn/kg 24 h prior to the Mt assay, and the other mouse received no treatment. The Cd removal step was carried out once, twice or 3 times to estimate the loss of Mt during the treatment with Hb. Although the estimated value of gross Mt decreased as the number of Cd removal step increased, net Mt values showed a good agreement. This result suggests that Mt is not lost by the treatment with Hb and heating at 100°C. The number of the treatment with Hb has no effect on the estimation of Mt.

Eaton and Toal¹⁵⁾ have reported that the repeated treatments with Hb caused a slight under estimation of Mt. They considered that the effect was due to a stripping of Cd from Mt by repeated treatments with Hb. It is unclear why the present result was different from that of Eaton and Toal. However, total amount of Hb added may be concerned because they applied equal volume of 100 μl of 2 % Hb solution, while we added various volumes of hemolysate to equalize the total amount of Hb. In any case, it is of no use to repeat

the treatment with Hb, because only a single treatment with Hb is enough to determine the Mt content accurately. We recommend only a single treatment with Hb for the Cd-hem method.

4. Conclusion

Bovine and swine hemolysates are satisfactorily available for the Cd-hem method instead of the rat hemolysate. Although any Mt is not lost by the treatment with Hb and heating at 100°C, only a single treatment is recommended because it is enough for the removal of Mt-unbound Cd. For the accurate determination of Mt, the value of blank which is determined without biological samples should be subtracted from that of biological samples because the Mt-unbound Cd cannot be removed completely by this treatment even though the treatment is repeated 3 times.

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