Hepatic distribution of iron, copper, zinc and cadmium-containing proteins in normal and iron overload mice

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Abstract Subcellular distribution of metal-containing proteins of Fe, Cu, Zn and Cd were determined in the liver samples of iron overload mice by size exclusion high performance liquid chromatography with on-line coupling to UV and inductively coupled plasma mass spectrometry. Collision cell techniques was used to remove polyatomic interferences for some elements, such as Fe. Comparative molecular weight (MW) information of the elemental fraction was obtained within a retention time of 40 min. Fe was present only in high-MW (HMW) protein; Cu, Zn and Cd were found in different MW proteins. It was also observed that these four elements studied showed predominant association with HMW fractions. Moreover, compared with the normal group, we found that the contents of these elements except Cu significantly increased and the distribution of some elements like Cd changed in iron overload mouse liver. It means that excessive iron accumulation in vivo may affect the metabolism of other element such as Zn and Cd.

Keywords Iron overload · Metalloproteins · Hepatic distribution · SEC-HPLC-ICP-MS

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Introduction

Research into trace element speciation in biological samples is nowadays accepted as one of the most dynamic research areas in metallomics. Different elements in proteins or protein complexes have different bioactivity. Essential elements are especially important for many biological detoxification and metabolic processes, and toxic elements may adversely affect these processes while present in relatively high amounts (Roberts 1981; Zaidi et al. 2002). The function of many metalloproteins critically depends on their interaction with a metal, usually a transition one, such as Cu, Fe, Zn (Szpunar 2005; Lobiński et al. 2006). Many metalloproteins such as metallothioneins (MT) involve in many pathophysiological processes including metal ion homeostasis and detoxification, protection against oxidative damage, cell proliferation and apoptosis

(Nordberg 1998; Miyazaki et al. 2002), and others serve within an organism as transporters of essential nutrient ions contaminants and metal probes.

Iron serves an essential role in life. However, excesses of iron are toxic. Iron overload is one of the most common metal-related toxicities. The liver which performs many metabolic functions is the principal target for iron toxicity because it is mainly responsible for taking up and storing excessive amounts of iron (Papanastasiou et al. 2000). Our previous studies have identified that administration with total of 500 mg/kg iron-dextran significantly increased the hepatic iron content of mice (Zhao et al. 2005; Zhang et al. 2006). However, the form of the increased iron and the alteration of other elements of nutritional and toxicological interest have not been studied.

Inductively coupled plasma mass spectrometry (ICP-MS) has been widely used for element specific detection since the 1980s. Because of its isotope specificity, versatility and high sensitivity, it becomes a molecule-specific technique when applied as a detector in chromatography (Sadi et al. 2002; Martino et al. 2002; Ferrarello et al. 2002), capillary (Kannamkumarath et al. 2002; Yeh and Jiang 2002) and planar electrophoresis (Chery et al. 2003). Moreover, an increasing number of reports indicated that ICP-MS becomes an attractive partner of electrospray and matrix-assisted laser desorption/ionization-MS for the investigation of biological matrices (Szpunar 2000; Szpunar and Lobiński 2002; Wind and Lehmann 2004). At present, the overwhelming majority of speciation analysis is developed using ICP-MS coupled to size exclusion chromatography (SEC). SEC is a well-established protein fractionation technique, which is frequently utilized for the separation of fairly large proteins and polypeptides. It can separate the various elemental species of the same or different elements as cleanly as possible before introduction to detecting instruments. Recently, a large number of valuable reports had used SEC-ICP-MS for probing metal–protein complexes. SEC-ICP-MS was used to acquire elemental profiles in cytosols of Alzheimer diseased brains (Richarz and Bratter 2002). A similar technique confirmed that both iron and zinc were bound to the enzyme human endothelial NO synthase (Leber et al. 1999). De Smet et al. (2001) also used SEC-ICP-MS to study the dynamics of (Cd, Zn)-MT in gills, liver and kidney of common carp during exposure to cadmium.

In this paper, SEC with on-line UV and ICP-MS detection was used to study metal-containing species in the liver samples of iron overload mice. The metals of interest including iron (Fe), copper (Cu), zinc (Zn) and cadmium (Cd). Gentle extraction conditions at physiological pH were chosen to minimize changes to the proteins during the analysis.

Materials and methods

Materials and reagents

Iron-dextran and Blue-dextran were purchased from Sigma-Aldrich Chemical Co. A 0.05 mol l^{-1} Tris mobile phase solution was prepared by dissolving solid Tris base in 1,000 ml of water and adjusted to pH 7.7 with diluted HCl solution. Cytochrom C, hemoglobin, ovalbumin, albumin and transferrin were obtained from Sigma and used for calibration of molecular weight (MW) by SEC. Other analytical reagents were purchased from local market. Deionized water (Milli-Q, Millipore, Bedford, MA, USA) was used for preparation of all solutions. All solutions were filtered through a 0.22-μm filter before used.

Animal treatment and preparation of subcellular constituents

Ten male Kunming mice, purchased from Tongji Medical School, Huazhong University of Science and Technology (China), initially weighing 20.63 ± 0.52 g, were used. Mice were randomly divided into two groups. One group received five doses (one dose every 2 days) of 100 mg Fe/kg each (i.p., 20 mg Fe/ml iron-dextran saline solution), the other group received the same volume of saline as a control. All five mice were housed in a large plastic cage and given free access to food and tap water. The animals of both groups were kept at 25 ± 2°C under a 12-h dark/light cycle. Animal care in this study conformed to the NIH Guide for Care and Use of Laboratory Animals (NIH publication 86-23, revised 1986).

After 45 days from the last iron-dextran injection, mice were fasted overnight. They were anesthetized with ethyl ether. The liver was quickly removed and weighed, then perfused with 4°C saline to exclude the blood cells and finally blotted on filter paper. After

that, the liver was weighted and homogenized in nine volume of ice-cold 50 mmol l^{-1} phosphate buffered saline. The homogenate was centrifuged at $1,000g$ for 15 min at 4°C in a Hermle Z323K centrifuge. After removing the precipitation, the obtained supernatant was centrifuged at $10,000g$ for 10 min at 4°C in an Avanti-J30I Beckman ultracentrifuge. In this step, the pellets were mitochondrial fractions. Subsequently, the supernatant was transferred into another centrifugal tube and centrifuged again at $105,000g$ for 90 min at 4°C. The microsome fraction and the cytosol were collected from precipitation and the supernatant, respectively. Finally, 50 mmol l^{-1} phosphate buffers were added to wash the rough mitochondrial and microsome fraction, respectively. Half of each subcellular fraction from liver sample was dried for trace element assay, and the other half was digested by nitric acid and hydrogen peroxide. The digested sample was used to analyze total element contents. Protein concentration was measured as Peterson (1977) described.

Instrumentation

For chromatographic separation, the Protein-Pak SW Glass column was connected to a Waters metal-free 626 pump equipped with a Rheodyne model 7725i manual injector (50 µl). A waters 2487 dual λ absorbance detector was used on-line with the high performance liquid chromatography (HPLC) equipment for detection of protein elution. ICP-MS experiments were done on a Thermo Elemental X7, quadruple-based instrument. For on-line metal monitoring, the outlet of the HPLC column was connected to ICP-MS by PEEK tubing, and the sensitivity of the ICP-MS instrument was optimized using collision cell techniques (CCT). The detailed analytical conditions were shown in Table 1. Possible polyatomic interferences in the analytical solutions were considered, which can be removed by using CCT mode.
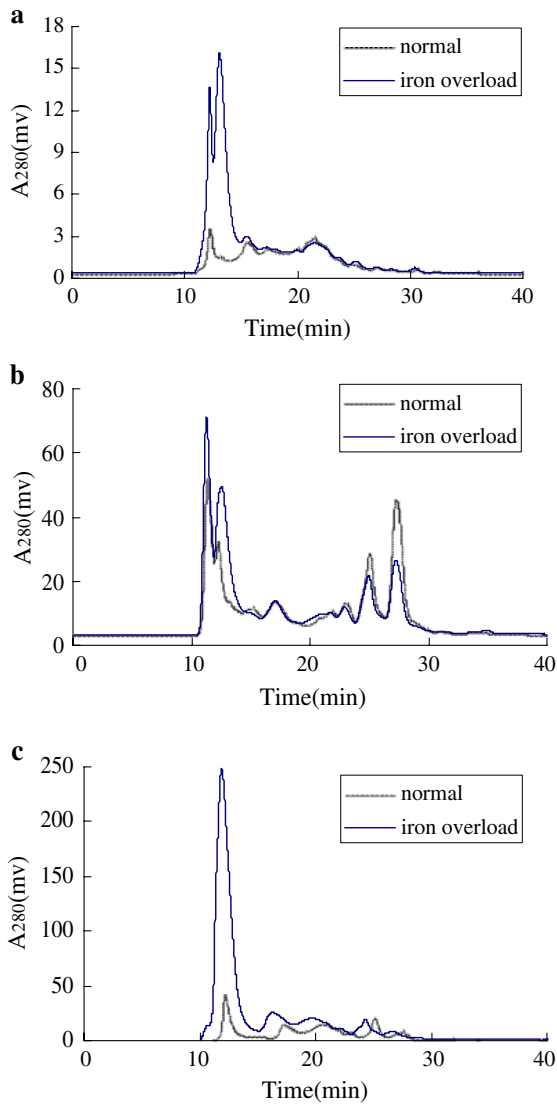
Results

Profiles of proteins in liver homogenate by SEC with on-line UV detection

The chromatograms obtained from supernatant (a), mitochondria (b) and microsome (c) of mouse liver homogenate are shown in Fig. 1. From Fig. 1, we can see that there are several separable protein fractions in different subcellular compartments and the most abundant fractions are at relatively short retention time. The question of which fractions contain specific

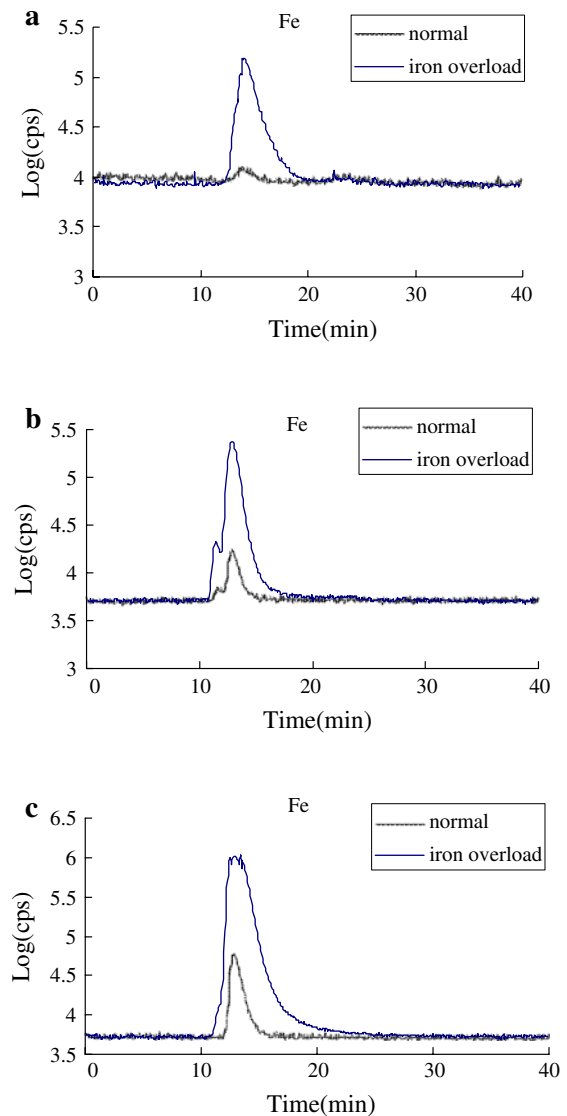| Table 1 Size exclusion chromatography and ICP-MS instrumental conditions		
SEC conditions		
Column	Protein-Pak 200SW Glass Column 10 μ 8 × 300	
Molecular weight range	10,000–300,000 Da	
Mobile phase	50 mmol l^{-1} Tris–HCl (pH = 7.7)	
Flow rate	0.5 ml/min	
Injection volume	50 µl	
Column temperature	Room temperature	
UV wavelengths studied	254, 280 nm	
ICP-MS conditions		
Forward RF power	1,200 W	
Plasma Ar flow rate	13.0 l/min	
Auxiliary Ar flow rate	0.7 l/min	
Nebulizer Ar flow rate	0.84 l/min	
Sampling depth	122 mm	
Dwell time	500 ms	
Isotopes monitored	^{54}Fe, ^{57}Fe, ^{65}Cu, ^{66}Zn, ^{68}Zn, ^{111}Cd	
Total analysis time	2,400 s	
CCT mode		
Component of He–H$_2$	7.28% H$_2$ (v/v)	
He–H$_2$ flow rate	7.5 ml/min	

Fig. 1 Typical SEC-ICP-MS chromatograms obtained from supernatant (**a**), mitochondria (**b**) and microsome (**c**) of normal and iron overload mouse liver homogenate. Other conditions were given as described in Table 1

Fig. 2 Chromatograms for Fe in supernatant (**a**), mitochondria (**b**) and microsome (**c**) of normal and iron overload mouse liver homogenate. Other conditions were given as described in Table 1

elements of Fe, Cu, Zn and Cd requires ICP-MS, which replaces the UV detector for the results, was shown in Figs. 2–5. In this experiment, recovery was high (>90%) for each element, suggesting that the SEC column has retained virtually no metalloproteins.

Fractionation of iron-containing proteins

Typical chromatographic profiles of Fe from liver homogenate are shown in Fig. 2. In the present
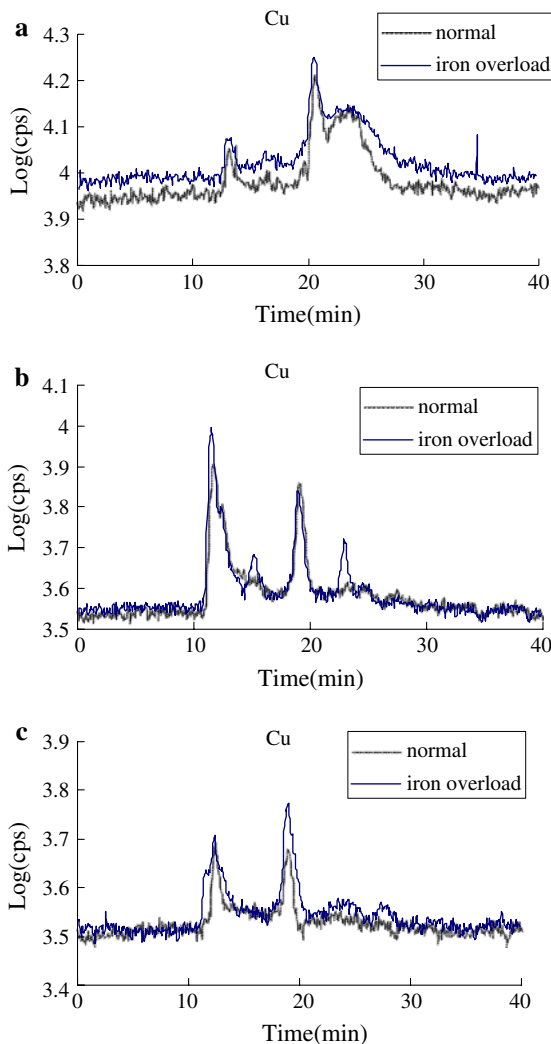
experiment, either in normal or iron overload mouse liver, Fe has one sharp chromatographic peak at retention time of about 12.8 min (MW > 300 kDa), but there was another peak at the retention time of about 11.5 min only in mitochondria. In addition, compared with normal group, we found the content of Fe was significantly increased in all liver subcellular fractions, especially in supernatant and microsome, and the peak area increased about 34 and 28 times, respectively. This result was similar to that reported

by Parkes and Templeton (Parkes and Templeton 1994), in which they also found iron overload can cause a proportionally high deposition of iron in the hepatic microsomal and cytosolic compartments.

Fractionation of copper-containing proteins

Figure 3 shows the typical chromatographic profiles obtained for Cu. From Fig. 3, we can see that copper is presented by three peaks both in normal and iron overload mouse liver supernatant: one at retention time of about 11.5 min is a high molecular weight

Fig. 3 Chromatograms for Cu in supernatant (**a**), mitochondria (**b**) and microsome (**c**) of normal and iron overload mouse liver homogenate. Other conditions were given as described in Table 1

(HMW) protein (MW > 300 kDa), the second one with the relative MW is ∼40 kDa, and the third one which is consecutive with the second one. There are two peaks in normal liver mitochondria with MW about >300 and ∼40 kDa, but there are four peaks in iron overload liver homogenate mitochondria, two of which are also included in the normal ones. The retention times of the other two peaks in iron overload liver mitochondria are 15.4 and 23.1 min, and the relative MW are ∼42 and ∼24 kDa, respectively. However, there are only two peaks both in normal and iron overload liver microsome, the relative MW are >300 and ∼34 kDa, respectively.

Fractionation of zinc-containing proteins

The chromatograms of Zn from liver homogenate are shown in Fig. 4. This element was found to be distributed differently among three or five MW fractions in different liver subcellular fractions. There are two main chromatographic peaks both in normal and iron overload liver supernatant, and the retention time are 12∼13 min (MW > 300 kDa) and 21∼22 min (MW ∼30 kDa), respectively. However, in the mitochondria and microsome of iron overload mouse liver, especially in the microsome, the proteins containing Zn were mainly present in the form of HMW species. Compared with the normal group, we found that the content of Zn was significantly increased in iron overload mouse liver, and the most predominantly increased form was HMW.

Fractionation of cadmium-containing proteins

Figure 5 shows the typical chromatogram for ^{111}Cd in liver extract. In present work, Cd-containing species could not be detected in supernatant, but could be detected at low spectral resolution both in mitochondria and microsome. From Fig. 5, we can see that two chromatographic peaks were separated in mouse liver mitochondria and microsome: one is in a HMW fraction with the retention time about 13.0 min (MW ∼70 kDa), the other is in a LMW fraction with the retention time about 23.4 min (MW ∼24 kDa). Moreover, we also found that the content of ^{111}Cd significantly increased both in iron overload liver mitochondria and microsome, but the forms were different. The increased ^{111}Cd in mitochondria
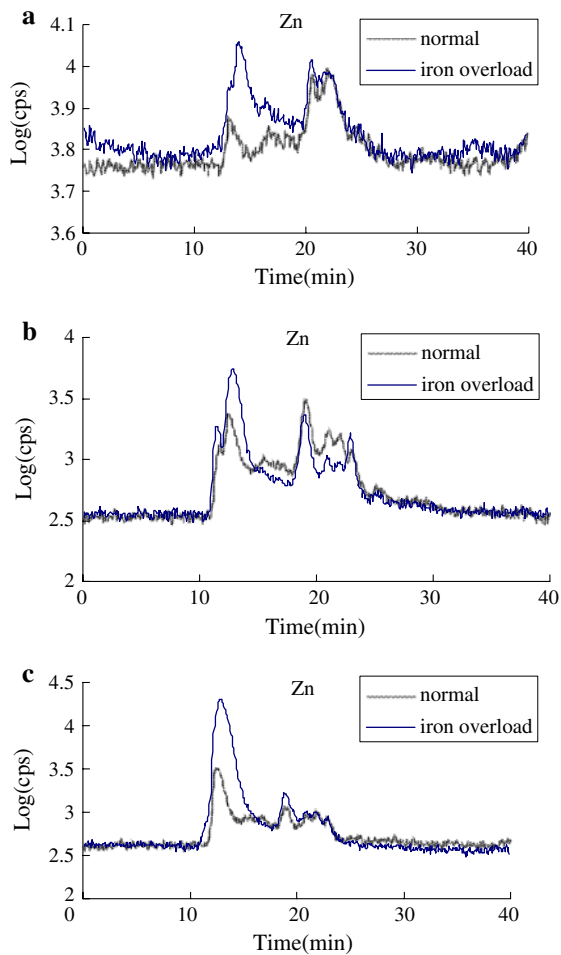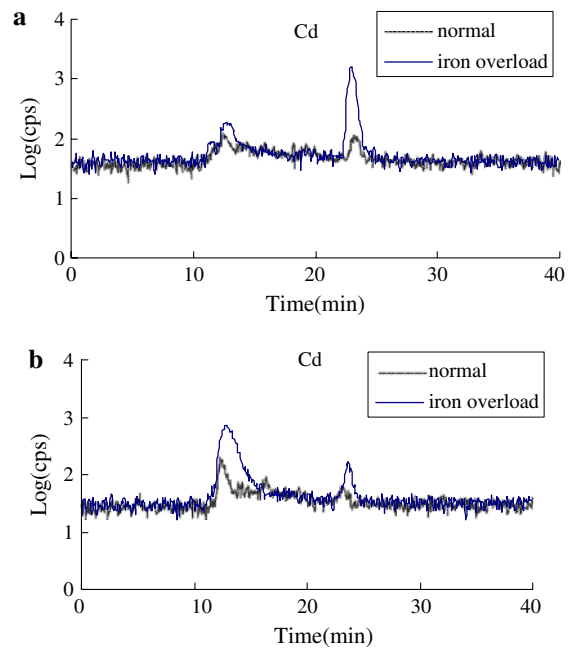
Fig. 4 Chromatograms for Zn in supernatant (**a**), mitochondria (**b**) and microsome (**c**) of normal and iron overload mouse liver homogenate. Other conditions were given as described in Table 1

existed only in LMW fraction, but in microsome, existed only in HMW fraction.

Total element concentrations in mouse liver subfractions

The total element concentrations were tested by SEC-HPLC-ICP-MS both in normal and iron overload mouse liver subfractions, the results are shown in Table 2. It can be seen that the content of Fe was significantly increased in iron overload mouse liver, especially in supernatant and microsome, the content increased about 29 and 22 times, respectively, while only about nine times of increased in liver mitochondria. In addition, we can see the total content of Cu

Fig. 5 Chromatograms for Cd in mitochondria (**a**) and microsome (**b**) of normal and iron overload mouse liver homogenate. Other conditions were given as described in Table 1

did not show any conspicuous difference in these two groups, but the concentration of both Zn and Cd were significantly increased, especially in mitochondria and microsome. These results further certified our conclusion that the excessive iron may be change the metabolism of some elements in liver.

Discussion

In present work, in order to obtain an accurate relative MW in liver homogenate of iron overload mice, the SEC column was calibrated with several standard proteins, and the chromatograms were recorded by UV-Vis absorption at 280 nm. The concentration of each protein was 1 mg/ml. Blue-dextran (2,000 kDa) was used for determination of the void volume; the elutions of other proteins are based on their own MW, each with a certain retention time. A calibration curve was established between the retention time and MW of protein. In addition, the chromatographic separation conditions were also evaluated for several different mobile phases in this work (data not shown). Finally, we found the best

Table 2 Total element concentrations in normal and iron overload mouse liver analyzed by ICP-MS

Subcellular	Fe (μg g^{-1}) Normal	Fe (μg g^{-1}) Iron overload	Cu (μg g^{-1}) Normal	Cu (μg g^{-1}) Iron overload	Zn (μg g^{-1}) Normal	Zn (μg g^{-1}) Iron overload	Cd (μg g^{-1}) Normal	Cd (μg g^{-1}) Iron overload
Supernatant	168.5 ± 0.3	4845.1 ± 3.1***	12.8 ± 0.04	13.9 ± 0.2***	112.2 ± 0.9	133.7 ± 2.5***	0.01 ± 0.004	0.01 ± 0.006
Mitochondria	523.1 ± 0.7	5050 ± 6.8***	25.5 ± 0.3	24.6 ± 0.2	44.9 ± 1.5	96.2 ± 2.2***	0.06 ± 0.003	0.11 ± 0.001***
Microsome	748.7 ± 2.3	16986.1 ± 18.1***	15.9 ± 0.2	14.3 ± 0.08***	28.8 ± 1.9	72.2 ± 1.3***	0.06 ± 0.005	0.11 ± 0.007***

Values are mean ± SD ($n = 3$)

*** $P < 0.001$ compared with normal

Table 3 Protein contents in different subcellular fractions

Sample	Protein content (mg/ml)		
	Supernatant	Mitochondria	Microsome
Normal	11.72 ± 1.4	11.32 ± 0.6	8.04 ± 2.1
Iron overload	13.16 ± 0.8	10.45 ± 1.1	10.63 ± 0.5

Values are mean ± SD ($n = 3$)

resolution and minimal retention time were obtained when using 50 mmol l^{-1} Tris–HCl at pH = 7.7.

The protein concentrations of all samples determined according to Peterson (1977) were close to each other (Table 3), and the injection volume of each sample was the same. However, from Fig. 1, it seems that the protein concentration of iron overload sample is much higher than that of normal sample. The reason may be due to that the protein concentration presented in Fig. 1 were obtained from the absorbance at 280 nm, excessive iron in ferritin may increase the absorbance at 280 nm which would give us a false result. This presumption was confirmed in another experiment, in which we found the absorbance of 1 mg/ml ferritin at 280 nm was higher than that of 1 mg/ml hemoglobin (data not shown).

Accumulation of excessive iron mainly occurs as complexes with transferrin, ferritin, hemoprotein and hemosiderin in liver. In order to determine the distribution of Fe in these molecules, we monitored two stable isotopes, ^{54}Fe and ^{57}Fe. CCT mode is used for avoiding the possible ^{40}Ar^{16}O$^+$, ^{40}Ar^{17}Cl$^-$, ^{38}Ar^{16}OH$^+$ interferences for this element. By means of chromatographic separation of protein and detection by ICP-MS, Stuhne-Sekalec et al. (1992) studied excessive iron accumulation in human and animal tissues and reported the distribution of iron in healthy rat liver and iron overload human liver, the sensitivity and spectral resolution were similar to the present work.

In order to investigate if iron overload will affect speciation of other trace elements in liver, different SEC-ICP-MS chromatograms were observed for several elements (Cu, Zn and Cd) in iron overload mouse as well as normal ones. The present results indicated that the copper content and distribution have no significant change in mouse liver supernatant and microsome (Fig. 3), this result was similar to that of Faa et al. (2002). However, we found that the copper distribution in iron overload liver mitochondria has a

noticeable change when compared with normal ones. It means that iron overload has not any dominant impact on hepatic copper content, but can change the distribution of Cu in mouse liver, especially in mitochondria. Wang et al. (2001) found that most of copper exists in the smaller protein fractions in bovine liver extraction. But in our experiment, we found that proteins which contain copper are different in different mouse liver subcellular fractions. In supernatant, most of the copper existed as LMW protein, and this result was similar to that of Wang et al. (2001), but in the mitochondrial fraction, most copper existed as HMW protein, while in the microsomal fraction, these two types were equivalent. In contrast to copper, the study showed that excessive iron in vivo has little influence on the hepatic zinc distribution, but can increase zinc protein content in mouse liver (Fig. 4). The probable reason may be that excessive iron caused the overexpression of some regulatory proteins which can mediate both Zn and Fe or some zinc-containing proteins, such as MT. Liuzzi et al. (2006) found that a transporter called Zip14 over-expressed, which was abundantly expressed in hepatocytes, could increase the uptake of both Zn and Fe. It also reported that hepatic MT in chicks was significantly increased after administrated with Fe (McCormick 1984; Fleet et al. 1990). Many studies have indicated that the accumulation and toxicity of Cd in vivo are related with nutritional status of the organism, especially with trace element, such as Fe, Zn and Ca (Jurczuk et al. 1997; Tanaka et al. 1995). Our results indicated that iron overload has prominent impact not only on the content of cadmium, but also on its distribution in mouse liver (Fig. 5). The probable reason may be that the type of MT which binds with Cd is different in liver mitochondria and microsome. Therefore, with the increasing of expression of MT induced by excessive iron, the forms of increased Cd-containing proteins are different. It has been reported that increased iron uptake would also increase Cd uptake (Raja et al. 2006), here we further confirmed that higher iron status in living body will also increase Cd uptake.

It was concluded that iron accumulation in vivo could affect the metabolism of other elements, such as Cu, Zn, Cd. We have shown the relationship between excessive iron and other elements (Cu, Zn, Cd). However, the elemental speciation by SEC-ICP-MS is only an initial step in identifying and charactering the metalloprotein associated with their fractions. The SEC fractions obtained should be a mixture of proteins with similar molecular size, a further separation in these fractions is still ongoing in our laboratory. In future, it is necessary to identify the metal-binding proteins in iron overload mouse liver by other multidimensional separation techniques, and to elucidate the possible role of excess iron in influencing the activities and metabolisms of other elements.

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