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Preparative Isolation of Adult Human Liver Metallothionein Isoforms

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Preparative human liver metallothionein (MT) isolation is described. MT was saturated with cadmium to follow MT purification spectrophotometrically instead of by metal content and to increase the stability of the protein. A concentrated, MT-rich fraction of the liver cytosol was prepared by selective organic solvent (acetone or acetone/methanol) fractionation. Conventional gel filtration and ion-exchange chromatographies resolved two MT isoforms, MT-I and MT-II. When needed, purification of MT from other low-molecular weight proteins was further increased by gel filtration chromatography at zero ionic strength, i.e., in distilled water. Reversed-phase high performance liquid chromatography of both MT isoforms resolved further peaks sharing MT properties not only from the MT-I but also from the MT-II ion-exchange isoform. The results show that it is feasible to perform a human liver MT isolation from an entire human liver with a reasonable laboratory capability.

Key words: Metallothionein, Human liver, Preparative isolation, Reverse-Phase HPLC.

Metallothionein (MT) is a unique low-molecular weight, cysteine-rich, heavy metal-binding protein widely distributed in nature. Although its actual physiological function remains to be established, it appears that MT could be related to Zn metabolism (2) and/or could function as an efficient antioxidant (6, 18, 19).

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By conventional methods (1) two isoprotein fractions differing slightly in amino acid composition are resolved in most species. By accepted nomenclature, they are referred to as MT-I and MT-II (12). However, it is now well-accepted that the number of isoforms is higher in primates. Human liver MT has been found to be comprised of six distinct isoforms, five of which were characterized as subspecies of the MT-I isoform (8). These MT isoforms were resolved by the use of reversed-phase high-performance liquid chromatography

(RP-HPLC), which has demonstrated its superior capability in resolving MT isoforms not detected using conventional techniques.

Previous human MT isolations have used just a few grams of tissue. Preparative human MT isolations would require, however, to use the entire liver (2-5 kg of tissue). In the present work it has been attempted to develop a methodology that enable us to work with an entire human liver with reasonable laboratory capabilities.

Materials and Methods

HPLC equipment.— A Bio-Rad liquid chromatograph system provided with two pumps, a high pressure mixer, an automation module, a controller module, a valve assembly with either 100 µl or 2 ml sample loop and complete software was used. UV absorbance (215 or 250 nm) was monitored with the Model 1306 monitor and conductivity with the normal Bio-Rad conductivity monitor. A Bio-Rad reverse phase (RP 318) column (10 × 250 mm) was used.

Human liver 1.— A 5 kg human liver was frozen within 12 h *post mortem* and stored at −20° C until use. For MT isolation, the liver was put in 4 l of ice-cold 10 mM Tris-HCl, pH 8.6, containing 10 mM 2-mercaptoethanol. Before complete thawing, the liver was cut into small pieces and homogenized in 200-250 g portions in a Waring blendor with 1 vol. (v/w) of ice-cold buffer (10 mM Tris-HCl, pH 8.6, containing 10 mM 2-mercaptoethanol and 0.25 M sucrose). The homogenate was centrifuged immediately at 16,000 g for 25 min at 4° C in a GSA rotor. The cytosol thus obtained was maintained continuously at 4° C. Cadmium was added when amounts of approximately 1.5 l of cytosol were obtained to get a final concentration of 25 µg Cd/ml cytosol. This will assure a complete saturation of metallothionein with cadmium (except when copper is present in the protein). The cytosol obtained (5.5 l), were left overnight at 4° C. The presence of both 2-mercaptoethanol and cadmium assures that MT is not oxidized.

The next day, the cytosol was subjected either to acetone selective fractionation or to acetone/methanol selective fractionation. In the former case, ice-cold acetone was added to cytosol with stirring to give 60 % acetone and centrifuged (16,000 g, 10 min, 4° C); the pellet was discarded and the supernatant was made up to 80 % acetone and the proteins were allowed to precipitate overnight at 4° C. In the latter case, ice-cold acetone was added to cytosol with stirring to give 60 % acetone and centrifuged as before. The pellet was discarded and to the supernatant ice-cold methanol was added (v/v) and subsequently more acetone was added (v/v) to the acetone/methanol solution and the proteins were allowed to precipitate overnight at 4° C.

Proteins precipitated by either acetone alone or acetone plus methanol were recovered differently. In the case of the acetone fractionation, the proteins were firmly sticked to the bottom of the flasks and therefore no new centrifugations were needed. In the case of the acetone/methanol fractionation, a strong floculation of the proteins instead of stickment to the walls was present. Thus, the entire volume had to be centrifuged (16,000 g, 10 min, 4° C) to collect the proteins. Proteins were redissolved in both cases in small volumes (150-400 ml) of 10 mM Tris-HCl, pH 8.6, at room temperature, centrifuged and the final supernatant stored at −10° C.

Protein solutions were concentrated by liophylization, thawed, made up 20 mM 2-mercaptoethanol, centrifuged (16,000 g, 10 min, 4° C), filtered and applied to a Sephadex G-50 (Fine) column (2.6 × 120 cm) equilibrated with 10 mM

Tris-HCl, pH 8.6. Elution was done with the equilibration buffer at a flow rate of 40 ml/h at 4° C, and 6.7 ml fractions were collected. Absorbance at 230, 250 and 280 nm was monitored. The low molecular-weight proteins were further purified by DEAE-Sephadex A-25 ion-exchange chromatography. Samples were applied without prior concentration to a column (2.3 × 14 cm) pre-equilibrated with 20 mM Tris-HCl, pH 8.6, and eluted with a continuous gradient as described in the text at room temperature. Fractions were collected and absorbance monitored; cadmium, zinc and copper content were measured by atomic absorption spectrophotometry.

For further purification, MT peaks from the ion-exchange chromatography were concentrated by liophylization and applied to a column (2.6 × 120 cm) of Sephadex G-50 equilibrated in H_2O. After elution with H_2O at 4° C, MT peaks were frozen and liophylized. For final isolation, MT fractions were applied to a semi-preparative reverse phase HPLC column (10 × 250 mm). The procedure followed was essentially that of RICHARDS and STEELE (14). Buffer A consisted of 10 mM sodium phosphate, pH 7.0, and buffer B consisted of 60 % acetonitrile in buffer A. MT was eluted with a two-step, linear gradient usually consisting of 0-10 % B for 0-10 min followed by 10-20 % B from 10-100 min. The column was maintained at room temperature and run at a flow rate of 2 ml/min. MT was dissolved in buffer A, filtered, and applied to the column through the loop. After sample injection, buffer A was run at least 5 min before starting the gradient. The elution was monitored continuously at either 215 or 250 nm. In addition, the eluted proteins were collected (1 fraction/min) and the absorbances, measured. After each run the column was washed with 100 % B.

Human liver 2.— A 3 kg human liver was similarly obtained and processed as the first liver, although in fewer days because of its smaller size. Thus, the liver was partially thawed, homogenized, centrifuged and the cytosol selectively fractionated with acetone after saturation of MT with cadmium. The 60-80 % acetone precipitated proteins were redissolved in buffer, and applied to a Sephadex G-75 (Superfine) column (5 × 145 cm) equilibrated and eluted with the Tris-HCl buffer. The MT peak was further purified by DEAE-Sephadex A-25 ion-exchange chromatography. MT-I and MT-II were stored frozen. For HPLC runs, MT isoforms were injected directly to the reverse-phase column.

Materials.— Water and acetonitrile were HPLC quality; all other chemicals were at least reagent grade. Sephadex G-50 and G-75 were from Pharmacia (Uppsala, Sweden).

Results and Discussion

The use of selective acetone fractionation for MT isolation from other species is a standard procedure (5, 21). Figure 1 shows the Sephadex G-50 chromatography from the 60-80 % acetone precipitated proteins of the human liver 1. The profile of the acetone/methanol isolation was essentially the same (not shown). Therefore, because there is no need of centrifugation to collect the precipitated proteins and these are more easily redissolved, we conclude that the acetone fractionation is a superior choice compared to the acetone/methanol fractionation. As expected, the low molecular-weight protein peak showed a high absorbance at 250 nm, typical feature of the binding of cadmium to MT (9). This peak was further purified by DEAE-Sephadex ion exchange chromatography (fig. 2). A complex pattern of peaks, which were different depending upon the wavelength studied, was obtained. Three peaks with high absor-

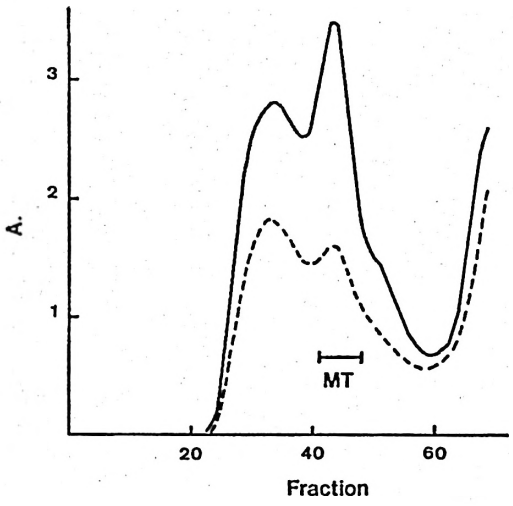

Fig. 1. *Sephadex G-50 chromatography of the 60-80 % acetone precipitated proteins from human liver 1.*

Only protein-containing peaks are shown. The column (2.6 × 120 cm) was eluted at a flow rate of 40 ml/h with 10 mM Tris-HCl, pH 8.6, at 4° C. The collected fractions (6.7 ml) were monitored at 250 (___) and 280 (- - -) nm.

bance at 250 nm were obtained. As expected, all of them were rich in cadmium. However, only one of them, MT-II, was eluted after starting the gradient. MT-I was eluted during the washing of the column. We have previously reported such a behavior for rat MTs (5), which was thought to be the consequence of the use of ammonium acetate buffer instead of the commonly used Tris-HCl. Overloading of the column could be responsible for the early elution of human MT-I, although further work is needed to elucidate this question. In any case, human MT-I was eluted by the gradient if the column was washed with 10 mM Tris instead of 20 mM (see below). As expected, cadmium replaced most of the zinc from the MT molecules under the conditions used in this study (7).

A peak sharing similar properties with MT-I and MT-II, that is, high absorbance at 250 nm (indicating the presence of mercaptide bonds) and low at 280 nm (indicating the absence of aromatic amino acids) and high content of cadmium, eluted first during the washing of the column. This peak was also rich in copper in addition to cadmium compared to MT-I and MT-II. Native zinc, copper-MT has been detected and isolated in normal human fetal livers (15, 16). The use of 2-mercaptoethanol was reported to be needed for an extensive recovery of this form of the protein. Since cadmium is able to displace zinc but not copper from MT (3), and we have used 2-mercaptoethanol during the isolation, the cadmium, copper-rich peak eluted first by the column washing is likely to be a mixture of the MT isoforms, early eluted because of the presence of copper in the molecule. However, no further studies of the identity of this protein(s) have been made in the present work.

The study of the different absorbances and metal contents of the eluted proteins clearly indicated the existence of contaminating proteins, especially in the case of the MT-II containing peak. This was fully illustrated when we increased the resolution of the DEAE-Sephadex chromatography by changing the gradient and the flow rate (fig. 3). A protein rich in aromatic amino acids eluted closely to MT-II. Therefore, further purification was needed.

We have previously reported that rat MT aggregates in the absence of ionic strength, i.e., in distilled water (5), aggregation that was not due to molecular oxidations since it was not affected by the presence of 2-mercaptoethanol. Therefore, we attempted to further purify the MT isoforms obtained by DEAE-Sephadex chromatography by subjecting them to Sephadex G-50 chromatography in H_2O. Figure 4 clearly shows that both human MT-I and MT-II isoforms eluted as high-molecular weight proteins, indicating aggregation of the MTs as it was the case

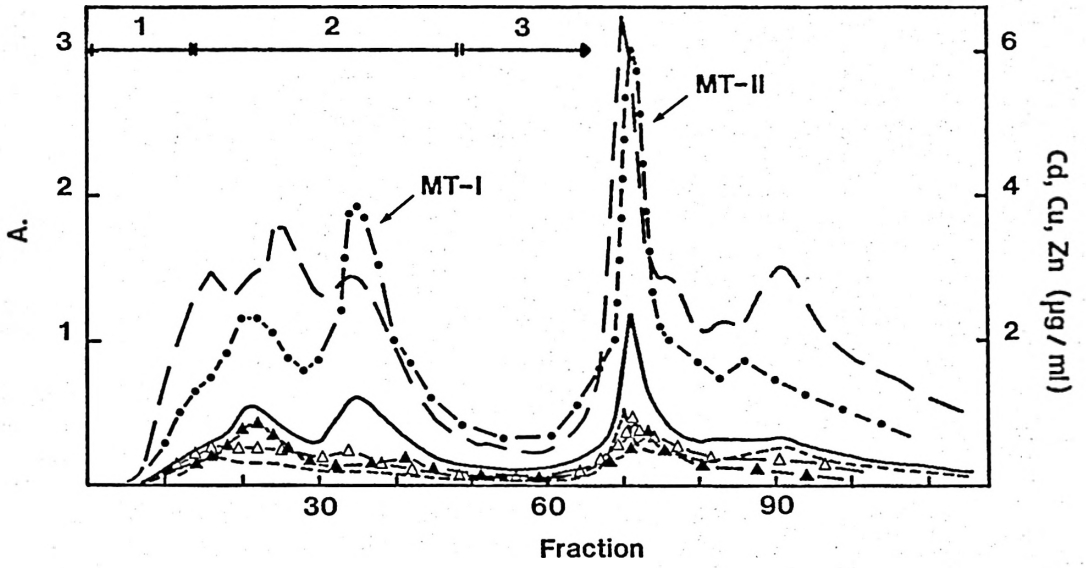

Fig. 2. *DEAE-Sephadex A-25 ion-exchange chromatography of the protein found in the low-molecular weight peak from fig. 1.*

Proteins were applied without concentration to the column (2.3 × 14 cm), which was pre-equilibrated with 20 mM Tris-HCl, pH 8.6. After extensive washing, a gradient from 20 to 300 mM (200 ml each) was used to elute the sample at a flow rate of 60 ml/h. Fractions (5-6 ml) were collected during sample application, 1, and column washing, 2, in addition to during the gradient, 3. Absorbances at 230 (_ _), 250 (___) and 280 (_ _ _) nm were measured, and the levels of cadmium (●_ _ _ _●), copper (▲_ _ _▲) and zinc (△_ _ _ _△) determined.

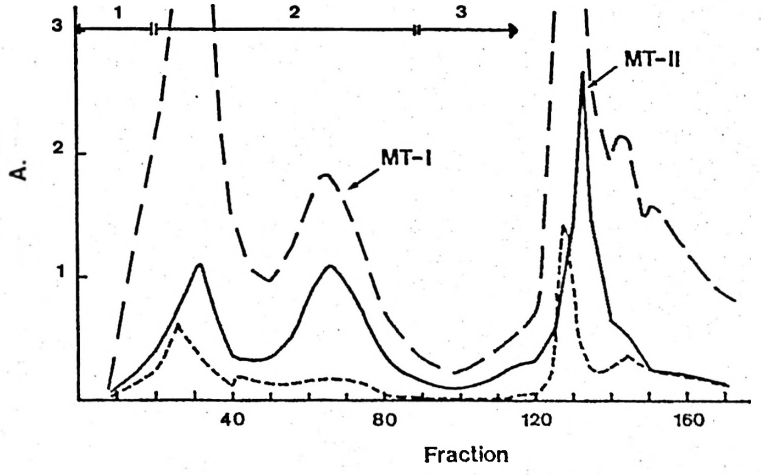

Fig. 3. *DEAE-Sephadex A-25 ion-exchange chromatography of the proteins found in the low-molecular weight peak from the Sephadex G-50 chromatography of the acetone/methanol isolation.*

In this case a gradient from 20 to 200 mM (200 ml each) was used to elute the sample at a flow rate of 23 ml/h. Absorbances at 230 (_ _), 250 (___) and 280 (_ _ _ _) nm were measured.

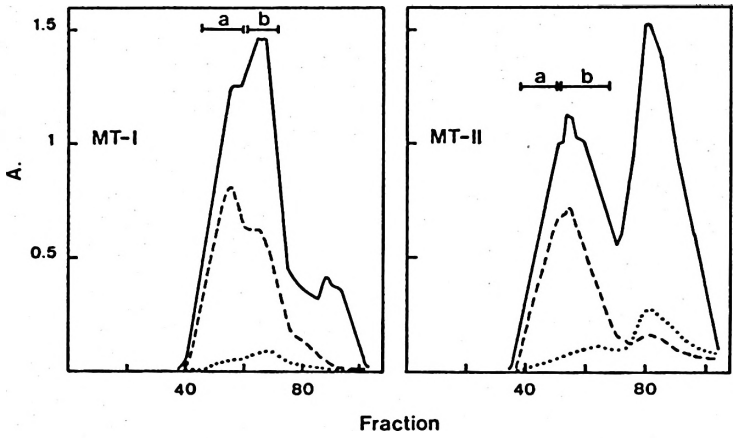

Fig. 4. *Sephadex G-50 chromatographies in distilled water of the human liver MT-I and MT-II isoforms isolated by ion-exchange chromatography (see fig. 2).*
The column (2.6 × 120 cm) was eluted at a flow rate of 50 ml/h at 4° C. The collected fractions (4.2 ml) were monitored at 230 (___), 250 (_ _ _) and 280 (.. .. ..) nm.

with rat MTs (5). In contrast, the contaminant protein(s) rich in aromatic amino acids eluted as expected as a low molecular-weight protein. The MT-I profile showed a greater heterogeneity than MT-II, which is not surprising taking into account that MT-I is comprised of several subspecies as resolved by reverse-phase HPLC, whereas MT-II is a single species (8). MTs at this stage displayed a typical absorptium spectrum (11), i.e., the absorbance at 280 nm was very low and a shoulder at 250 nm was present (not shown). In any case, human MT-I and MT-II were each separated into two pools (fig. 4) for HPLC studies.

Figures 5a and 5b show the reverse-phase chromatographies of the two pools of MT-I, a and b, obtained in the Sephadex G-50 chromatography in H_2O. The HPLC profiles for both pools were essentially the same, suggesting that the differences observed between pools in the 230:250:280 nm wavelength ratios could be due to different metal (cadmium, zinc, copper) ratios in the protein. Figures 6a and 6b show that the same was true for the two pools of MT-II (figure 4).

At least six human MT isoforms have been isolated by reverse-phase HPLC (8). By conventional ion-exchange chromatography, two MT isoforms, MT-I and MT-II, are obtained. That designation obeys to the order of elution of the isoforms in the ion-exchange chromatography, eluting MT-I first and MT-II later. However, the elution of the MT isoforms in reverse-phase HPLC is just the opposite. Thus, when the MT-II isoform obtained by ion exchange chromatography is chromatographied by reverse-phase HPLC it elutes earlier than the MT-I isoforms (8). On the other hand, reverse-phase HPLC resolved five isoforms in the MT-I obtained by ion-exchange chromatography, whereas MT-II seemed to be comprised by a single isoform.

Figures 5 and 6 show the reverse-phase chromatography of the MT-I and MT-II ion-exchange isoforms, respectively. In agreement with previous data (8), five MT-I isoforms (characterized by a 250/280 ratio greater than 20) were obtained, and a major peak eluting before than the MT-I isoforms was obtained in the MT-II chromatography. However, other much

smaller peaks were also present in the MT-II reverse-phase chromatography sharing similar characteristics to the large peak (i.e. 250/280 ratio greater than 20). This is

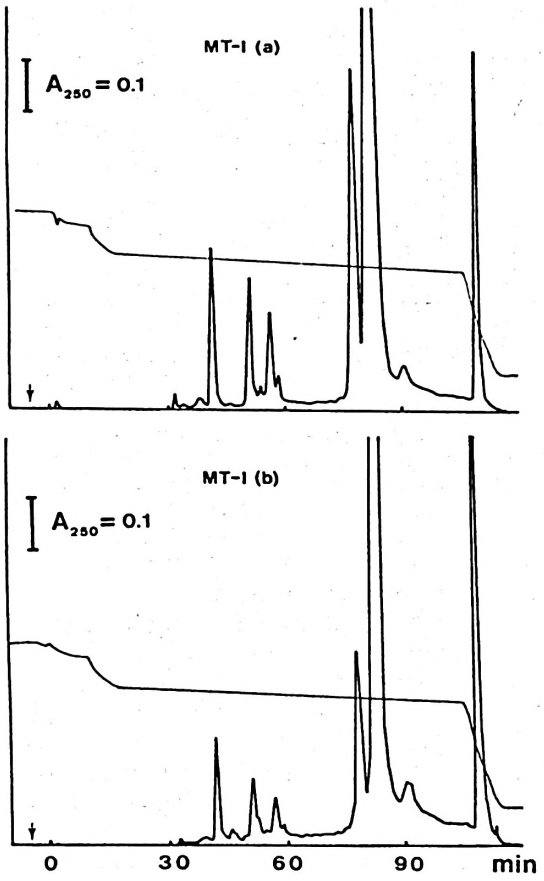

Fig. 5. *Reversed-phase high-performance liquid chromatography (RP-HPLC) of the pool a and b, respectively, from the human liver MT-I chromatography in H_2O (see fig. 4).*
At the arrow, the sample was injected through the loop. The separation of the isoMTs was performed with a Bio-Rad RP 318 column and a two-step linear gradient of 0-10 % B for 0-10 min followed by 10-20 % B from 10-100 min at ambient temperature and at a flow rate of 2 ml/min. Buffer A was 10 mM sodium phosphate, pH 7.0, and buffer B was 60 % acetonitrile in buffer A. The effluent from the column was continuously monitored for absorbance at 250 nm, and for conductivity (transversal line) which indicated the shape of the gradient.

in contrast to previous results (8), where a single MT-II isoform was obtained. This discrepancy could be explained by the fact that we have used a semi-preparative HPLC column instead of an analytical one, allowing us to see peaks because of the higher sample loading that otherwise would be ignored. However, the physiological significance of these peaks is not clear. Although human MT isoforms are encoded by a family of genes consisting of about 14 members, only six functional human MT genes have been characterized to date (4, 10, 13, 17, 20). It seems unlikely that those extra peaks were artifactual, since that was not the case with the MT-I isoforms on the one hand (and they were obtained with the same process), and we have made a reverse-phase HPLC with rat MT-II isolated similarly (5) obtaining a single peak (data not shown), in agreement with previous data (14), on the other hand.

Human MT isolation from liver 2 was considerably easier than that of liver 1. Its smaller size shortened the process, and for unknown reasons the presence of contaminant proteins in the 60-80 % acetone precipitate was much smaller than in the first liver. Thus, the 250/280 ratio in the MT peak of the gel filtration chromatography was about 7 in this case compared to 2 in the first liver. The ratio was much improved after the ion-exchange chromatography (greater than 14), and therefore the gel filtration chromatography in H_2O was not needed. In this case, both MT-I and MT-II, which were fully resolved, were eluted by the gradient (data not shown). Figure 7 shows the reverse-phase chromatography of the MT peak from the Sephadex G-75 chromatography. In this case, all the human MT isoforms are present, and the chromatogram is very similar to the ones previously reported (8). As it can be seen, there are three major isoforms. As expected, the first peak eluted by the HPLC gradient corresponded to the ion-exchange chromatography MT-II,

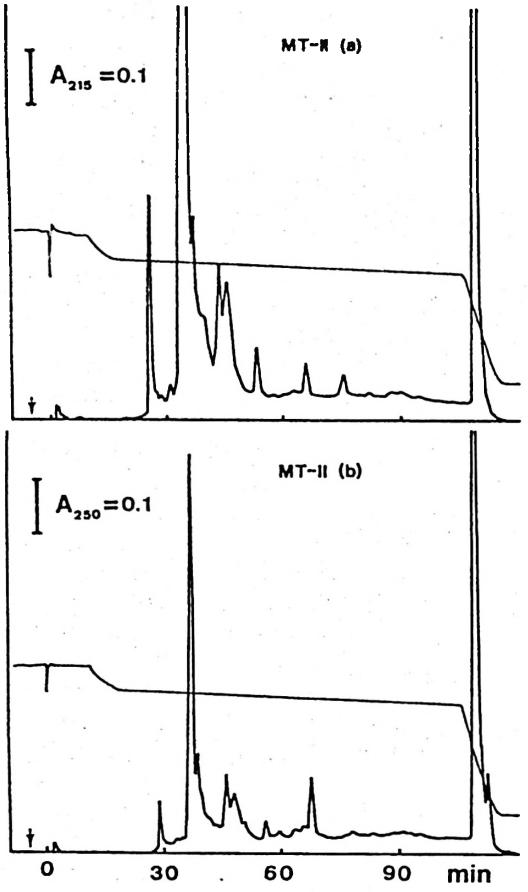

Fig. 6. *RP-HPLC of the pool a and b, respectively, from the human liver MT-II chromatography in H_2O (see fig. 4).*
The chromatographies were made as described in fig. 5.

whereas the later ones were the ion-exchange chromatography MT-I isoform (data not shown). Most of the HPLC smaller peaks eluted with the ion-exchange MT-II isoform, with retention times similar to those of the first human liver. In the case of the ion-exchange MT-I isoform, only 3 reverse-phase isoforms were readily detected. This was not surprising, since great changes in the relative proportions of the MT-I isoforms between livers have been detected (8).

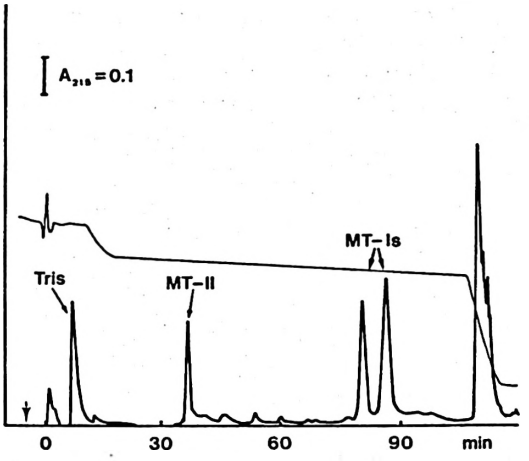

Fig. 7. *RP-HPLC of the proteins found in the low-molecular weight peak of the Sephadex G-75 chromatography of the 60-80 % acetone precipitated proteins from human liver 2.*
At the arrow, 100 µl of that peak were injected into the column. The chromatography was made as described in fig. 5.

As a final comment, we recommend to eliminate the phosphate and the acetonitrile from the MT isoforms obtained by reverse-phase HPLC as soon as they are collected, i.e. by passing them through a Sephadex G-25 column, since otherwise there will be problems in the reconstitution of the isoforms after freezing the solutions.

Acknowledgements

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Resumen

Se describe la purificación con fines preparativos de la metalotioneína (MT) de hígado humano total. La MT se satura con cadmio para seguir su purificación espectrofotométricamente, en lugar de por su contenido en metales, y para incrementar su estabilidad. Se prepara una fracción rica en MT del citosol hepático mediante el fraccionamiento selectivo con disolventes orgánicos (acetona o acetona/metanol).

Mediante el uso de las cromatografías convencionales de gel filtración e intercambio iónico se obtienen dos isoformas, MT-I y MT-II. La cromatografía de alta resolución en fase reversa de las dos isoformas de la MT resuelve más picos con típicas propiedades de MT de ambas isoformas. Los resultados muestran que se puede realizar un aislamiento de MT de un hígado humano entero con un utillaje de laboratorio razonable.

Palabras clave: Metalotioneína, Hígado humano, Purificación preparativa, HPLC en fase reversa.

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