Purification and Characteristics of Recombinant Mouse Metallothionein-I from *Escherichia coli*[1]

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The mouse metallothionein-I (mMT-I) cDNA was amplified by polymerase chain reaction (PCR), inserted into vector pGEX-4T-1, and expressed in *Escherichia coli* as a carboxyl terminal extension of the 26-kDa glutathione-S-transferase (GST). Analyzed by SDS-PAGE, the amount of the expressed fusion protein GST-MT was over 50% of total cellular proteins. After the fusion protein had been digested with thrombin on a Glutathione-Sepharose 4B affinity chromatography column, recombinant mMT-I was purified by gel filtration on Sephadex G50. The results of molecular mass, amino acid composition and sequence of 10 amino acids at the N-terminus of the recombinant mMT-I demonstrate that the purified protein is the one we desired. The ratios of metal:protein and thiol:protein are the same as those of wild-type MT. The half-dissociation pHs of Cd, Cu, and Zn from recombinant mMT-I were 3.57, 1.40, and 5.20, respectively, which are in agreement with those from native rabbit MT-I. The ultraviolet absorbance and circular dichroism (CD) spectra at pH 8.0 and pH 2.0 were all similar to those of native MT, indicating that they have the same metal-thiolate structure even though six amino acid residues have been added at the N-terminus of the recombinant protein.

Key words: characteristics, *Escherichia coli*, expression, metallothionein and mouse.

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Metallothioneins (MTs) are a family of cysteine-rich, low molecular weight, metal-binding proteins that are widely found from single-cell organisms to humans (*1*). They are traditionally considered to be involved in cellular detoxification of heavy metals such as cadmium and mercury, as well as in the homeostasis of essential metal ions, *e.g.*, zinc and copper (*2*). They are also important in mammalian UV response, serving as scavengers of free hydroxyl radicals or sources of zinc for DNA repair enzymes (*3*). MTs are usually single-chain proteins, without an α-helix or β-sheet, and bind a total of seven equivalents of bivalent metal ions. Thus MTs are ideal proteins for the examination of structure/function relationships by site-directed mutagenesis or other molecular biological methods.

A number of systems have been used in *Escherichia coli* to express MT directly or as a fusion protein (*4-6*), but attempts to produce high level of recombinant proteins achieved only limited success because of their low stability and the toxicity of high content of thiol-groups (*7*). Since our work in this laboratory is focused on the relationship between structure and function of MTs, we need to establish an efficient expression system to produce MT and MT analogues. On the other hand, as native MT is difficult to get from animals, recombinant MT will take its place in medical treatment or other areas. So it is useful to find another way to produce large quantities of recombinant MT and MT analogues.

In this study, we describe the expression of mouse MT-I cDNA as a carboxyl terminal extension of glutathione-S-transferase in *E. coli*, followed by the purification and characterization of the recombinant protein.

MATERIALS AND METHODS

Bacterial Strains and Plasmids—Plasmid pBX/MT, containing two copies of mouse MT-I cDNA between *Bam*HI sites, was kindly provided by Dr. Richard D. Palmiter. Fusion expression vector pGEX-4T-1 and *E. coli* strain BL21 were purchased from Pharmacia Biotech. Plasmid construction was made in *E. coli* strain DH5α, and gene expression was performed in *E. coli* strain BL21. Both plasmid-containing strains of *E. coli* were grown in Luria-Bertani (LB) medium supplemented with ampicillin (80 μg/ml).

In Vitro Amplification, Cloning, and Sequence Analysis of Mouse MT-I (mMT-I) cDNA—Two oligonucleotide primers were synthesized as follows: 5′-CCGAATTCACG-ATGGACCCGAAC-3′ (primer 1) and 5′-CCGTCGACTCA-GGCACAGCA-3′ (primer 2). Plasmid pBX/MT was used as a template for the polymerase chain reaction (PCR). Reactants were subjected to 30 cycles as follows: 93°C for 1 min (denaturation), 55°C for 1 min (annealing), and 72°C for 2 min (extension), using a DNA Thermal Cycler 480 (PEKIN ELMER CETUS). The PCR products were analyzed by electrophoresis on 8% polyacrylamide gel, then digested with *Eco*RI and *Sal*I and ligated into the corresponding sites of pGEX-4T-1, the glutathione-S-trans-

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Abbreviations: IPTG, isopropyl-β-D-thiogalactoside; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

ferase (GST) fusion expression vector. The sequence of the cloned mMT-I cDNA in pGMA8 was verified by sequencing in both directions using PCR oligonucleotide primers.

Expression and Purification of Expressed mMT-I Protein—An overnight culture of *E. coli* BL21 containing pGMA8 was diluted 1 : 10 (v : v) in fresh LB medium and grown at 37°C for 1 h, then metal ions (Cd or Zn) and IPTG were added to final concentrations of 0.5 mM and 0.1 mM separately to induce expression of fusion proteins. After incubation for a further 4 h at 37°C, cells were pelleted by centrifugation at 6,000 rpm for 15 min and resuspended in 5% of the original volume of PBS (PBS: 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3) within 3 mM β-mercaptoethanol, then lysed by mild sonication at 4°C. Triton X-100 was added to a final concentration of 1% and the suspension was mixed gently at room temperature for 1 h to facilitate solubilization of proteins. Fusion protein was purified from supernatant by single-step affinity chromatography on Glutathione-Sepharose 4B (Pharmacia) and digested with thrombin while it was associated with the gel at 25°C (*8*). During the digestion, metal ions (Cd, Cu, or Zn) were added to a final concentration of 0.5 mM. The eluent containing thrombin and recombinant mMT-I cleaved from the fusion protein was fractionated on Sephadex G50. After metal concentrations had been determined by atomic absorption spectrophotometry, the portion corresponding to the recombinant mMT-I was lyophilized.

Extraction and Reconstitution—Rabbit MT-I was directly isolated and purified from the liver of rabbit which had been administered hypodermically with a large amount of $ZnSO_4$. The rabbit liver was homogenized, centrifuged, and precipitated with ethanol. MT-I was obtained by gel filtration on Sephadex G50, ion-exchange chromatography on DEAE-Sepharose Fast Flow, and desalination on Sephadex G25 (*9*). Preparation of apo-MT and reconstituted MT with Cd, Zn, and Cu were described previously (*10, 11*).

Characteristics of the Recombinant mMT-I—The content of protein was estimated using a Coomassie Brilliant Blue-based reagent (BIO-RAD). Concentrations of cadmium, zinc, and copper were determined by atomic absorption spectrophotometry using a Philips PU9200 model at 228.8, 213.9, and 324.8 nm respectively, and thiol-group content was analyzed using Ellman's reagent (5 mM DTNB, 1 mM EDTA, 6 M guanidine-0.1 M PBS, pH 7.3).

SDS-polyacrylamide gel electrophoresis was carried out as described previously (*12*) using a 15% polyacrylamide gel, and the percentage of the expressed fusion protein was estimated by ultraviolet thin-layer spectrophotometry. Molecular mass of the protein was determined by ion-spray mass spectrometry after oxidation of the protein with performic acid (*13*).

Amino acid composition was analyzed using a Beckman 121 MB analyzer after hydrolysis with 6 M HCl at 110°C for 24 h. Amino acid sequence analysis was carried out

a. ATG GAC CCG AAC TGC TCC TGC TCC ACC GGC GGC TCC TGC ACT
TGC ACC AGC TCC TGC GCC TGC AAG AAC TGC AAG TGC ACC TCC
TGC AAG AAG AGC TGC TGC TCC TGC TGT CCC GTG GGC TGC TCC
AAA TGT GCC CAG GGC TGT GTC TGC AAA GGC GCC GCG GAC AAG
TGC ACG TGC TGT GCC TGA

PCR primer 1: 5' CCGAATTCACGATGGACCCG*AAC 3'
PCR primer 2: 5' CCGTCGACTCAGGCACAGCA 3'

b.

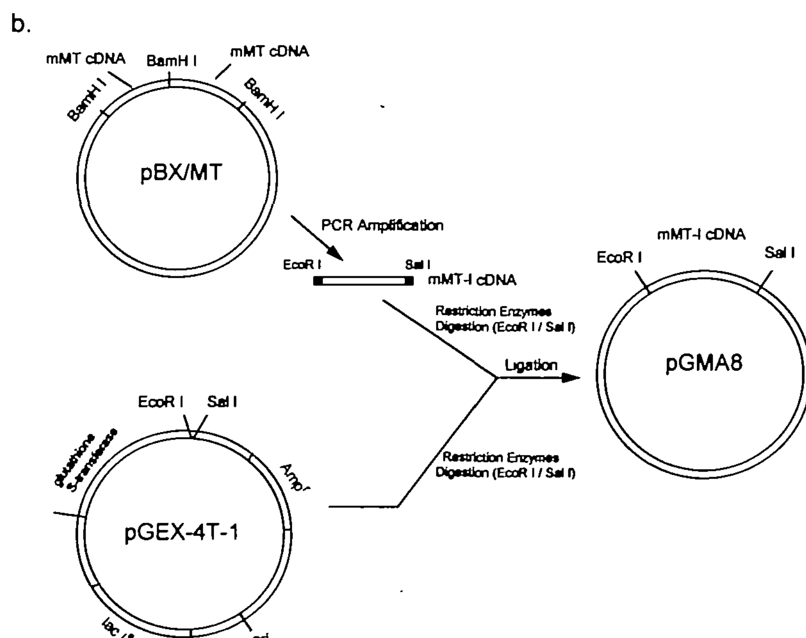

Fig. 1. **a: Sequence of mouse MT-I cDNA and the PCR primers** ["*" shows the replacement of codon (CCC) of Pro at position 3 with CCG]. **b: The mMT-I cDNA was inserted into pGEX-4T-1 between *Eco*RI and *Sal*I to generate the expression plasmid pGMA8.**

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after the protein had been subjected to 10 cycles of Edman degradation in an Applied Biosystems 491A protein sequenator.

Ultraviolet absorption spectra of proteins at pH 8.0 and 2.0 were recorded on a Shimadzu UV-240 spectrophotometer at room temperature with a protein concentration of 0.1 mg/ml. The circular dichroism (CD) spectra were measured with a Jasco J-500A spectropolarimeter in a quartz cell with a path length of 1.0 cm, and each spectrum reported was the average of four scans from 200 to 300 nm. Protein concentration was 6.5×10^{-5} M.

Analysis of metal-binding properties was performed as follows: 100-μl aliquots of purified MT (about 50 μg) in 50 mM Tris-HCl (pH 8.0) was incubated at room temperature for 1 h with 2 ml of buffer of different pHs (1.0-9.0), then applied to a Sephadex G25 column equilibrated with the same buffer. Metal ions eluted in the void volume (while binding with protein) and total volume were quantified and the proportion of the void volume was calculated.

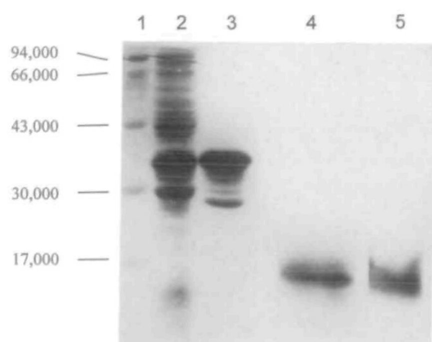

Fig. 2. **15% SDS polyacylamide gel electrophoresis analysis.** 1, molecular weight marker; 2, crude lysates of BL21 transformed with pGMA8; 3, GST-MT fusion protein after glutathione-Sepharose 4B; 4, purified recombinant mMT-I; 5, native rabbit MT-I.

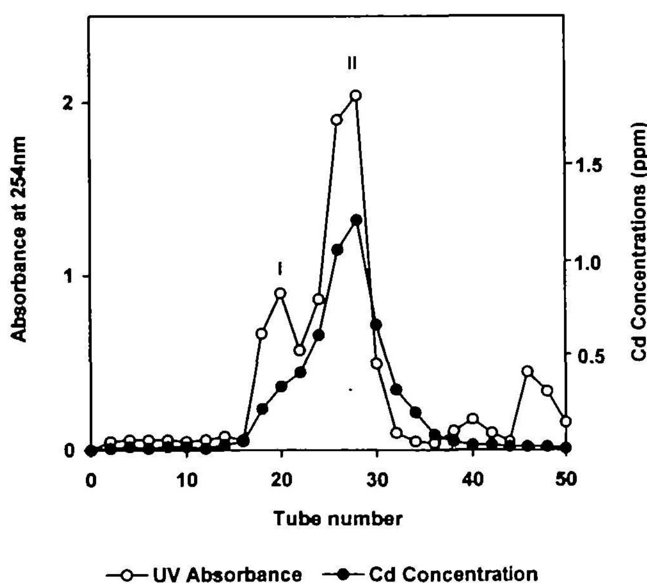

Fig. 3. **Gel filtration pattern on Sephadex G50 of eluent from glutathione Sepharose 4B after digestion with thrombin.** The UV absorbance was detected at 254 nm and the concentration of cadmium was determined by atomic absorption spectrophotometry.

RESULTS

Plasmid Construction—Mouse MT-I cDNA was amplified from pBX/MT by PCR using the two synthetic primers, in which the codon (CCC) for Pro at position 3 was replaced by CCG (Fig. 1a). After being verified by 8% polyacrylamide gel electrophoresis to have the predicted size (208 bp), the amplified products were inserted into expression vector pGEX-4T-1 to produce plasmid pGMA8 (Fig. 1b). The sequence of the cloned mMT-I cDNA was confirmed.

Expression and Purification of Recombinant mMT-I—Induction of the *tac* promoter with IPTG in cells transformed with pGMA8 results in the abundant synthesis (appox. 51% of total cellular proteins) of a 34-kDa protein consisting of glutathione-S-transferase and mMT-I (Fig. 2, lane 2). The GST-MT fusion protein was detected after affinity chromatography on Glutathione Sepharose 4B (Fig. 2, lane 3). The purified recombinant mMT-I and the native rabbit MT-I have the same mobility as shown in Fig. 2 (lane 4 and 5, respectively). Figure 3 shows the pattern of gel filtration on Sephadex G50 and reveals that cadmium is associated with peak II, which corresponds to the recombinant mMT-I released by thrombin.

Characteristic of Recombinant mMT-I—The amino acid composition (Table I), amino acid sequence at the N-terminus and molecular mass of the recombinant mMT-I were determined to confirm that the desired gene product was recovered from cell lysates. Of the 10 amino acid residues

TABLE I. **Amino acid composition of recombinant mMT-I isolated from *E. coli* cells.** Samples were hydrolyzed for 24 h at 110°C with 6 M HCl *in vacuo* and analyzed using a Beckman 121 MB analyzer. Values shown here were obtained from proteins oxidized with performic acid.

Amino acid	Recombinant MT (residues per mol)	Theoretical composition (residues per mol)
Asp	4.2	4
Thr	5.6	6
Ser	8.9	10
Glu	2.2	2
Pro	2.6	3
Gly	6.5	6
Ala	5.6	5
Cys	20.5	20
Val	1.9	2
Met	0.4	1
Ile	0.1	0
Leu	0.3	0
Phe	0.8	1
Lys	7.0	7
His	0.1	0
Arg	0.2	0
Total	66.9	67

TABLE II. **Content of metals and thiol groups of GST and recombinant mMT-I.** GST was prepared from cells transformed with pGEX-4T-1, and the recombinant mMT-I was purified from GST-MT after digestion with thrombin. Concentration of Cd was determined by atomic absorption spectrophotometry and thiol group content was assayed with Ellman's reagent.

Protein	Cd:protein	Thiol:protein
GST	0.14:1	1.8:1
Recombinant mMT-I	6.50:1	17.4:1
Theoretical ratio	7:1	20:1

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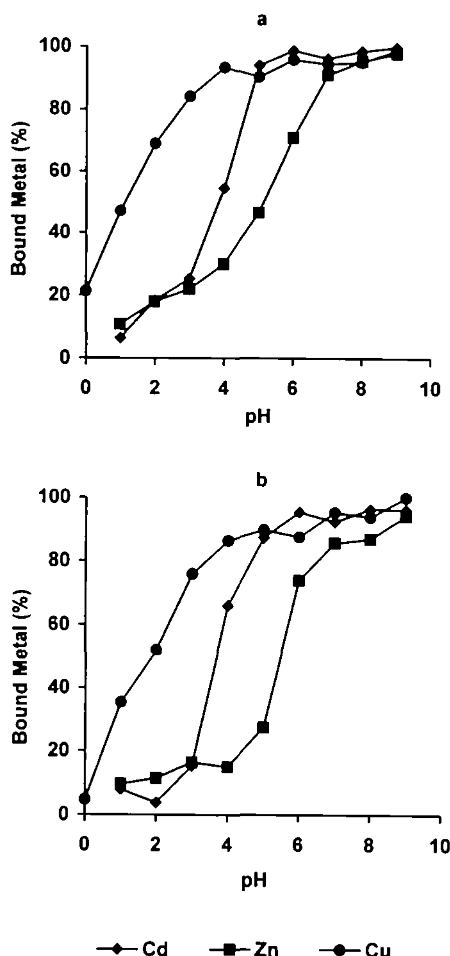

Fig. 4. Estimation of metal half-dissociation pH of recombinant mMT-I (a) and native rabbit MT-I (b). MT (about 50 μg) was incubated with 2 ml of buffer of different pHs (from 1.0 to 9.0), then applied to a Sephadex G25 column equilibrated with the same buffer. Metal ions eluted in the void volume and total volume were quantified. Data is shown as a proportion of metal bound. MTs binding with Cd, Zn, and Cu were reconstituted with the respective metal.

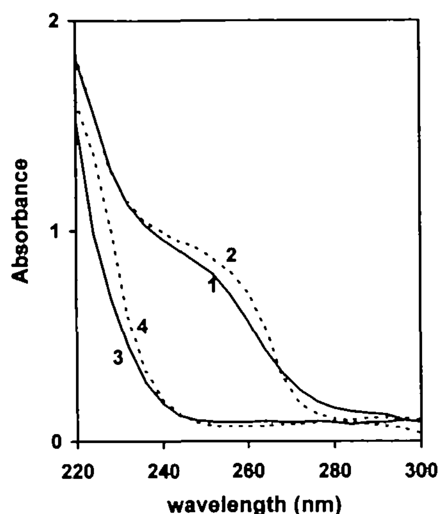

Fig. 5. Ultraviolet spectra of recombinant mMT-I and native rabbit MT-I. Protein concentration is 0.1 mg/ml. 1 and 3 refer to the recombinant mMT-I at pH 8.0 and 2.0, respectively; 2 and 4 refer to native rabbit MT-I at pH 8.0 and 2.0, respectively.

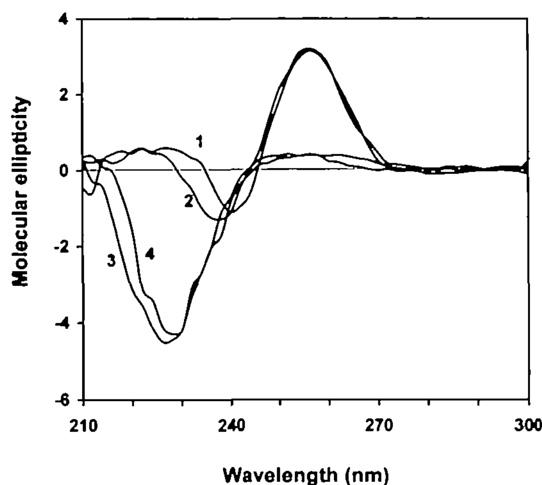

Fig. 6. Circular dichroism (CD) spectra of recombinant mMT-I and native rabbit MT-I. Protein concentration is 6.5×10^{-6} M. 1 and 3 refer to the recombinant mMT-I at pH 8.0 and 2.0, respectively; 2 and 4 refer to native rabbit MT-I at pH 8.0 and 2.0, respectively.

(^{N}Gly-Ser-Pro-Glu-Phe-Thr-Met-Asp-Pro-Asn) at the N-terminus, the first six are due to the digestion with thrombin from the C-terminus of glutathione-S-transferase. The molecular mass of the oxidized recombinant mMT-I is 7,200 Da. This is greater than that of native mMT-I (6,600 Da) because of the short peptide prior to methionine and the oxidation of cysteines to cysteic acids.

The contents of bound Cd and thiol groups of GST, recombinant mMT-I and native rabbit MT-I were measured (Table II). The data indicate that metals bind with the MT portion of the fusion protein. The pHs of half-dissociation of Cd, Cu, and Zn from recombinant mMT-I, purified from *E. coli* cells while the respective metal ions were supplemented during the procedure, were estimated to be 3.57, 1.40, and 5.20 (Fig. 4). These are in agreement with those from *in vitro* reconstituted rabbit MT-I (3.67, 1.83, and 5.48, respectively).

Figure 5 shows the similar ultraviolet absorption spectra of the native rabbit MT-I and recombinant mMT-I at pH 8.0 and 2.0. At pH 8.0, high cadmium-thiolate absorption at 250 nm and low absorbance at 280 nm are observed, while at pH 2.0 they both disappear. The CD spectra of the recombinant mMT-I are also very similar to those of native rabbit MT-I, as shown in Fig. 6. The absorption band at 260 nm, corresponding to the characteristic Cd-thiolate cluster at pH 8.0, diminished when pH was adjusted to 2.0.

DISCUSSION

The expression levels of MT in *E. coli* are always limited because its high content of thiol groups is toxic to the host cells. In this study, a fusion expression vector, pGEX-4T-1, was selected and a much higher yield was achieved than before. The GST-MT fusion protein synthesized in *E. coli* represents approx. 51% of total celluar protein, of which MT accounts for about 8.5%. The recovery of recombinant mMT-I from *E. coli* cells (3-4 mg per liter of culture) is

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considerably higher than the expression level achieved in *E. coli* (2 mg/liter) elsewhere (*7, 14*).

It has been reported that addition of supplementary metal ions to the medium during the expression could protect nascent recombinant MT from being attacked by proteinases in host cells (*15*). But in this work no significant difference in metal tolerance was observed between cells transformed with pGEX-4T-1 and pGMA8. The result is in disagreement with previous reports (*6, 7*), because mMT-I was expressed as a C-terminal extension of GST and thus could not bind metal and form the same metal-thiolate structure as it does when expressed individually.

The half-dissociation pH of metals is a criterion to distinguish MT from non-MT metal binding proteins. The same results from recombinant mMT-I and native rabbit MT-I also agree with those from horse equine MT reported previously (*16*). This means that the recombinant MT purified from *E. coli* is a mammalian MT, as we desired. Further experiments are required to determine whether the metal-binding properties of recombinant mMT-I are the same as those of the native protein.

The similar UV absorption and CD spectra of recombinant mMT-I and native rabbit MT-I suggest that the two proteins have the same metal-thiolate structure. The bands at 258 nm in the CD spectra must be attributable to the metal-thiolate chromophore, since aromatic acids and disulfide bridges are absent (*17*). The cotton effects disappear when the pH is lowered to 2.0 as a result of the dissociation of metal-thiolate cluster because all the cysteines are protonated. Furthermore, the fact that no α-helix or β-sheet is observed in the CD spectra indicates that the N-terminal region is flexible, as is that of native mammalian MTs. The similarity of the two proteins shows that the additional six amino acid residues at the N-terminal region do not change the formation of the two clusters, because the N-terminal region is mobile, not being involved in metal chelation (*18, 19*).

When we investigated the immunoreactivity of the recombinant mMT-I, we found that the elongated MT did not react with its monoclonal antibody either in Western blotting or ELISA. This means that the six amino acid residues at the N-terminus affect the immunoreactivity of recombinant mMT-I. It has been reported that the major antigenic epitopes of mammalian MTs are located in the N-terminal region together with the lysine-rich region around residues 20-25, which are remote in the primary sequence but proximal in the tertiary structure (*20, 21*). Although the N-terminal region is flexible, the elongation may change the conformation of this region and prevent it from approaching the lysine-rich region, thus causing the recombinant mMT-I to lose its immunoreactivity.

All these results show that an efficient expression system has been set up to produce mouse MT-I in *E. coli* and that the recombinant mMT-I has the same metal-binding structure and function as native rabbit MT-I except for its immunoreactivity. Further work is needed to investigate not only the immunoreactivity but also the structure and function after the additional amino acid residues are cleaved from the protein with cyanogen bromide.

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