Purification and Some Properties of Metal-binding Proteins in Housefly Larvae (Musca domestica)

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Cadmium and zinc-binding proteins similar to metallothionein have been isolated from housefly larvae (Musca domestica) exposed to cadmium chloride. Amino acid composition analysis found a high half-cystine content and an apparent minimum molecular weight of 5225. Metal-binding proteins of Musca domestica contained 3.9 g-atoms and 4.5 g-atoms of heavy metals per mole, respectively, and showed the spectral characteristic of cadmium-thionein, i.e., a broad shoulder at 250 nm and low residual absorption at 280 nm. The simple and specific replacement of cadmium and zinc bound to the protein with a cupric ion indicates that proteins have mercaptide bonding with a high affinity for copper. The molecular weight of the proteins modified with Ellman's reagent was 5300 ± 250 when measured by gel filtration in the presence of 6 M guanidine hydrochloride.

Metallothionein (MT) is a low molecular weight metal-binding protein rich in metals and cysteine residues but lacking aromatic amino acids. The occurrence of MT in both vertebrate and invertebrate tissue, as well as in microorganisms, has been well demonstrated.1

Insects are the most populous class of organisms in the animal kingdom. However, MT and the related metal-binding proteins in the Insecta class have been only studied in a small number of species. Maroni and Watson2 reported that cadmium-binding protein was induced by metals in the midgut of Drosophila melanogaster larvae. Debec et al.3 have found a protein that has the characteristic properties of MT in Drosophila cells grown in the presence of CdCl2. Everard and Swain4 identified MT in the stonefly (Eusthenia spectabilis). Yamamura et al.5 and Aoki et al.6 showed that Cd-binding proteins are inducible in midge larvae (Chironomus yoshimatsui) and in fleshfly larvae (Sarcophaga peregrina), respectively. In addition, Bouquegneau et al.7 confirmed that MT was also induced in the ileum of the cockroach (Blatella germanica) by Hg exposure. Detailed data such as amino acid composition is lacking for the metal-binding proteins in the insects mentioned above. However, Perry et al.8 showed that a 40-amino-acid protein in Drosophila is very similar in amino acid composition and nucleotide sequence to other known MTs.

As part of our studies to discover the differences between the species of insects and inducible metal-binding proteins, we have demonstrated that Musca domestica larvae commonly produce at least two metal-binding isoproteins upon CdCl2 exposure. In this paper we describe the purification and some properties of metal-binding proteins induced in Musca domestica larvae.

Materials and Methods

Reagents. Analytical grade chemicals and double-distilled water were used throughout the experiments. Sephadex G-75, DEAE-Sephaloc, and an electrophoresis calibration kit (low molecular weight proteins) were purchased from Pharmacia (Sweden). Rabbit liver MT-I, insulin B-chain, ribonuclease A, chymotrypsinogen A, ovalbumin, and bovine serum albumin were purchased from Sigma Chemical Company (U.S.A.).

Experimental animals. Second instar larvae of the housefly (Musca domestica strain S.R.S) were raised on moist compost (M powder rat diet, Oriental Yeast Co., Ltd., Tokyo) containing 1 mM CdCl2 for 24 h. The larvae were removed from the compost, washed thoroughly with distilled water, and air-dried.

Purification of metal-binding protein. The whole larvae were homogenized for 10 min at 0°C in three volumes of 10 mM Tris–HCl buffer (pH 8.6) containing 0.25 M sucrose and 100 mM 2-mercaptoethanol. After centrifugation at the homogenate at 10,000 rpm for 60 min at 4°C, the supernatant was further centrifuged at 4°C for 60 min at 100,000 x g to obtain soluble fractions. A 5-ml portion of the supernatant was put on a Sephadex G-75 column (2.6 cm i.d. x 70 cm) equilibrated previously with 10 mM Tris–HCl buffer, pH 8.6, and then eluted with the same buffer at a flow rate of 20 ml/hour. Five-milliliter fractions were collected. Concentrations of metals and molecular absorbances at 254 and 280 nm were measured on an atomic absorption spectrophotometer (Hitachi 170-30) and a spectrophotometer (dual path monitor UV-2, Pharmacia), respectively. The fractions V2/V0 2.3, which both had high absorbance at 254 nm and high metal content, were pooled and freeze-dried. This cadmium-rich fraction was concentrated to one-tenth of its original volume by ultrafiltration under N2 pressure using a YM-1 membrane (Amicon, Ireland) and put on a DEAE-Sephaloc column (1.3 cm i.d. x 25 cm) that was pre-equilibrated with 10 mM Tris–HCl, pH 8.6. The column was eluted with a gradient of 10 to 250 mM Tris–HCl buffer, pH 8.6. Two major peaks appeared, which were designated MBP-I and MBP-II, with intense absorption at 254 nm and residual absorption at 280 nm. Each peak contained high levels of Cd and Zn. The lyophilized sample of MBP-I and MBP-II were once desalted by a Sephadex G-25 column with 0.1 M ammonium carbonate and freeze-dried. MBP-I and MBP-II were rechromatographed on a DEAE-Sephaloc column with Tris–HCl buffer (pH 8.6) as the eluent with a gradient: Tris–HCl concentrations increased linearly from 10 to 100 mM and from 50 to 250 mM, respectively, to obtain purified protein.

Analytical methods and characterization of metal-binding protein. Protein was measured by the micro-biuret method9 and the ultraviolet absorption at 254 and 280 nm. Molecular weight was measured by the following two
Results and Discussion

Figure 1 shows the gel filtration profile obtained. The maximum contents, 1.8 μM of Cd and 0.7 μM of Zn, were detected in fraction 62, which also had the absorption maximum at 254 nm. Copper was present only as a trace and the data were omitted from the profile. Ve/V0 of fraction number 62 was 2.3 (rabbit liver MT-I was 2.0 on the same column), showing good agreement with the value of 2.2 to 2.5 for MT from midge larvae reported by Yamamura et al.5)

The fraction was put on a DEAE-Sepharose column pre-equilibrated with 10 mM Tris-HCl, pH 8.6, and elution was done with a 500-ml linear gradient.

Figure 2 shows the results of the anion exchange column chromatography. Two well-resolved major peaks appeared that had the characteristic absorption of cadmium-thionein, that is, an intensive absorption at 254 nm based on the cadmium-mercaptide binding and a residual absorption at 280 nm based on the lack of aromatic amino acids (A_{254}/A_{280}=8 or more) as mammalian MTs.17) Each peak contained high levels of Cd and Zn.

It is difficult to state if these two peaks represent true molecular species or are due to artificial modifications of a single metal-binding protein form. However, in general, MT produced by many animals consists of two forms, designated MT-I and MT-II, which can be separated from each other by DEAE-Sephadex chromatography.18) MBP-I and MBP-II were chromatographed with a 20 mM Tris-HCl (pH 8.6) at the flow rate of 0.5 ml/min on an Asahipak GS-320 column (50 cm) developed as gel filtration for HPLC. Contrary to the results from the DEAE-Sepharose column, MBP-II eluted first; the retention times of MBP-I and MBP-II were 19.2 min and 18.1 min, respectively. Kwohn et al.19) observed the same phenomenon for MT-I and MT-II isolated from striped dolphin and reported their retention times to be 18.8 min and 17.4 min, respectively. Furthermore, metal-binding proteins in midge larvae9) and fleshy larvae9) consist of a mixture of four or five isoproteins having several properties characteristic of MT. It is appropriate, therefore, to consider that the two peaks should correspond to iso-proteins of metal-containing proteins.

Fig. 1. Gel Filtration of Housefly Larvae Homogenate Supernatant on a Sephadex G-75 Column.

Supernatant (5.0 ml) was chromatographed on a 2.6 x 70 cm column with 10 mM Tris-HCl buffer (pH 8.6) at a flow rate of 20 ml/h. Cadmium (+ –), zinc (– + –), and absorbance at 254 nm (– – –) and 280 nm (–– –) were measured. Fractions (5 ml) were collected. The fraction denoted with a bracket was pooled for further processing.

Fig. 2. Chromatography of the Metal-binding Protein on a DEAE-Sepharose Column.

MBP fraction isolated by gel filtration was put on a DEAE-Sepharose column (1.2 x 25 cm) equilibrated with 10 mM Tris-HCl buffer, pH 8.6. Elution was done by a 500-ml linear salt gradient (limiting buffer 250 mM Tris-HCl, pH 8.6) at a flow rate of 60 ml/h. Fraction (7 ml) were collected. Cadmium (+ –), zinc (– + –), absorbance at 254 nm (– – –), and 280 nm (–– –) were measured.

Fig. 3. Polyacrylamide Disc-gel Electrophoresis of the Purified MBP-I.

MBP-I (5.0 μg) and marker proteins were put on 12.5% acrylamide gel and stained with Coomassie Brilliant blue R250.
Metal-binding Proteins in Housefly Larvae

![Graph showing partition coefficient against log molecular weight](image)

**Fig. 4.** Measurement of the Molecular Weight of MBP-I and MBP-II by Gel Filtration in 6 M Guanidine–HCl on Bio-Gel A-5m.

Partition coefficients, \( K_v \), were evaluated from the relationship \( K_v = (V_r - V_0) / (V_t - V_0) \). The marker proteins used are the following: (A), insulin B-chain; (B), ribonuclease A; (C), chymotrypsinogen A; (D), ovalbumin; (E), bovine serum albumin. A dotted line shows MBP-I and MBP-II from *Musca domestica*.

**Table I.** Amino Acid Composition of *Musca domestica* Larvae MBP-I

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Residues (%)</th>
<th>Residues Molecule</th>
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<tr>
<td>Asp</td>
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<tr>
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<td>0.2</td>
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<tr>
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<tr>
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<tr>
<td>Mol. wt.*</td>
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<td></td>
<td>5225</td>
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* Values obtained from 24 h hydrolysis of MBP-I.
* Percentage of total number of residues.
* Measured from the cysteic acid content in performic acid oxidized protein.
* Measured as methionin sulfone.
* Calculated as metal-free mol. wt.

Purified MBP-I was obtained by re-chromatography with a DEAE-Sephacel column as mentioned above. The protein was detected as a single band on SDS–PAGE and the molecular mass of its protein was estimated to be 14.5 kDa (Fig. 3). It was reported that, when examined using SDS–PAGE, MT or MT-like proteins behave as proteins with greater molecular weight.\(^{10}\) It is reasonable, therefore, to consider this protein to have a low molecular weight. The molecular weight of the metal-binding proteins from *Musca domestica* was 5300 ± 250; an average of the values obtained from gel filtration in the presence of 6 M guanidine hydrochloride (Fig. 4). The same value was obtained by gel permeation chromatography using HPLC.\(^{12}\) Calculation of the minimum molecular weight from the amino acid composition yields a value of 5225 which correlates well with the value measured by gel filtration.

Table I shows the amino acid composition of purified MBP-I. Virtually no aromatic amino acid residues were detected. This was also confirmed by the unusually low ultraviolet absorption in the 280 nm region. In comparison with mammalian MT, there is a lower proportion of cysteinyl residues.

**Figure 5** represents the ultraviolet absorption spectra measured from purified MBP-I. In this figure, an absorption band with a broad shoulder is seen in the region of 250 nm at pH 7.0, but disappears at pH 2.0, which is a typical pattern of cadmium-thionein (line A and B). The molar extinction coefficient of the protein was calculated as \(9.20 \pm 0.25 \times 10^3\), which is lower than the values of \(1.29 \pm 0.24 \times 10^4\) for stonefly MT\(^{40}\) and \(1.45 \times 10^4\) for equine metallothionein.\(^{15}\) Ultraviolet absorption spectra of MBP-II were similar to those of MBP-I.

Results of the metal analysis (Table II) indicate the presence of cadmium, zinc, and copper, as is frequently
observed in eukaryotic MTs. The metal levels were lower (3.7 g-atoms and 4.5 g atoms/mole protein, calculated as molecular weight 5300) than those previously reported for higher organisms.\textsuperscript{20} It is known that cadmium bound to MTs can easily be replaced simply by mixing with copper without affecting specifically coordinated metals to other ligands.\textsuperscript{15,16} and the replacement has been studied in detail in mammalian MTs.\textsuperscript{14,21,22} A result of the metal replacement reaction of this protein with cupric chloride indicates that cadmium and zinc were almost completely replaced by copper. The protein was separated by gel filtration and virtually no metal ion other than copper could be measured in the protein using atomic absorption spectrosocopy. The protein contained 3.95 g-atoms of copper, 0.02 g-atoms of cadmium, and 0.01 g-atoms of zinc.

The major absorption was in the far ultraviolet region with a weak shoulder at 270 nm attributable to copper charge transfer transitions (line C in Fig. 5). Absorption at 270 nm of the protein increased markedly with decreasing absorption at 254 nm. A shoulder appeared at 270 nm that showed a tendency dependent on the copper concentration (T. Kasai et al., unpublished paper). The ultraviolet spectrum of the copper-binding protein is similar to that of Cup-Ds isolated from yeast.\textsuperscript{23} The simple and specific replacement of cadmium and zinc bound to the protein with a cupric ion indicates that the protein has mercaptide bonding with a high affinity for copper.

Therefore, the metal-binding protein induced in Musca domestica larvae may be a metallothionein, because these properties mentioned above of this protein are characteristic of metallothioneins. It remains to be established whether or not this lower cysteine and metals content in comparison with mammalian MTs is an intrinsic property of this protein. Further work is in progress to confirm the amino acid sequence of this protein and the characterization of MBP-II.

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\textbf{References}