0163-4984/05/10600–0000 $30.00

Metallothionein-like Proteins and Zinc–Copper Interaction in the Hindgut of *Porcellio scaber* (Crustacea: Isopoda) Exposed to Zinc

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Received June 22, 2004; Revised August 9, 2004;
Accepted October 10, 2004

ABSTRACT

Metallothioneins (MTs) are ubiquitous low-molecular-weight metal-binding proteins, with a variety of functions in metal metabolism ascribed to them. Among terrestrial invertebrates, MTs have been studied in nematodes, insects, snails, and earthworms. The aim of this study was the characterization of MT-like proteins in the terrestrial isopod crustacean *Porcellio scaber* in order to analyze their probable role in the metabolism of copper (Cu) and zinc (Zn). Dietary Zn supplementation (793 µg Zn/g dry food, 6 d) was applied to stimulate MT synthesis. After separation of the hindgut postmicrosomic supernatant (cytosol) of Zn-exposed animals by gel filtration on a Sephadex G-75 column, a Cu- and Zn-containing peak was detected in the position of V_e/V_o~2, where MTs are expected to elute. Rechromatography of these fractions by size-exclusion chromatography–high-performance liquid chromatography revealed that the 215-nm absorbance peak coincided with the absorbance peak of the rabbit MT II standard. These low-molecular-weight Cu- and Zn-binding compounds, detected in the cytosol of the hindgut cells in Zn-exposed *P. scaber,* are considered to be Cu,Zn-MT-like proteins. To our knowledge, this is the first report on the characterization of MT-like proteins in isopod crustaceans.

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These results also indicate that both Zn and Cu dynamics in *P. scaber* hindgut are affected at the given dietary Zn supplementation and that MT-like proteins are involved in this Zn–Cu interaction.

Index Entries: Metallothionein; copper; zinc; *Porcellio scaber;* hindgut; digestive system; Crustacea.

INTRODUCTION

Metallothioneins (MTs) are ubiquitous proteins, characterized by low molecular weight (6000–7000 for the mammalian MTs), high metal content, characteristic amino acid composition (high cysteine content, no aromatic amino acids or histidine), typical distribution of cysteinyl residues, spectroscopic features characteristic of metal thiolates, and a tertiary structure dominated by metal–thiolate clusters that form at the metal-binding sites *(1,2).* A variety of functions in metal metabolism have been ascribed to MTs, but this issue still remains enigmatic *(3–7).* Metallothioneins have been isolated from a wide range of organisms. Concerning invertebrates, the index of entries in the Swiss–Prot protein sequence database (www.expasy.org/sprot) comprises descriptions of MTs in the following groups: Mollusca, Crustacea, Echinodermata, Diptera, Nematoda, and Ciliata. In addition, MTs were also characterized in earthworms *(8,9)* and in the springtail *Orchesella cincta (10).* Studies of MTs in terrestrial invertebrates have gained increasing attention in recent years *(11).* They might provide new insights into the biological diversity of MTs.

Concerning crustacean MTs, nine different MTs are described in the Swiss–Prot database. All of these reports refer to decapod crustaceans. Three different MTs, namely MT I, MT II and copper-specific CuMT-II, were isolated from the blue crab *(Callinectes sapidus).* In the mud crab *(Scylla serrata),* two MT sequences, MT I and MT II, were identified. The other four entries refer to MTs from *Homarus americanus, Astacus fluviatilis, Carcinus maenas,* and *Potamon potamios.* Crustacean MTs are known to be involved in the metabolism of essential metals (copper [Cu] zinc [Zn]) and also in binding of nonessential metal ions, such as cadmium *(12–17).* Investigations of MTs in the blue crab *(C. sapidus)* have indicated their role in the metabolism of Cu and Zn related to the molt cycle of the animals *(18–20).*

To our knowledge, the presence of MTs has not been yet demonstrated in the tissues of isopod crustaceans *(21–23).* The purpose of our investigation was the characterization of MT-like proteins in the hindgut of the terrestrial isopod crustacean *Porcellio scaber.* Because intestinal MT synthesis can be stimulated by dietary Zn supplementation in mammals *(24–26),* the animals were fed on Zn-dosed food (793 µg Zn/g dry food) and an elevated MT content in the hindgut was expected. The hindgut of isopod ~crustaceans extends from the foregut to the anus and is composed of a one-layered epithelium, lined with a cuticle *(27,28).* The apical and the

basal surfaces of the hindgut are in direct contact with ingested food and hemolymph, respectively. In this article, we report on the characterization of Cu,Zn-MT-like proteins in the cytosol of the hindgut cells in the isopod *P. scaber* fed on Zn-dosed food. The Zn–Cu interaction in the hindgut of Zn-exposed *P. scaber,* involving MT-like proteins and high-molecular-weight compounds, is further discussed.

MATERIAL AND METHODS

Animals

The specimens of *P. scaber,* Latreille 1804 (Crustacea: Isopoda) were collected in gardens in the vicinity of Postojna in southwestern Slovenia. Ninety males weighing between 38 and 131 mg were selected for the experiment. Females were excluded because of a possible interdependence of gravidity and the level of metal-binding compounds.

Food

The food mixture was prepared from food for laboratory mice and hazeltree *(Corylus avellana)* leaves in a 1 : 1 ratio. Zinc was added by soaking the food mixture with $ZnCl_2$ water solution. Nontreated (no Zn added) and Zn-dosed food were analyzed for Zn and Cu concentrations by radiochemical neutron analysis (RNAA) *(29).* Untreated food contained 142 µg Zn/g dry food and 16 µg Cu/g dry food. Zinc-dosed food contained 793 µg Zn/g dry food and 19 µg Cu/g dry food. The Zn concentration applied in Zn-dosed food was chosen on the basis of previous studies performed with *P. scaber (30–32)* and it was expected that it would not influence the feeding rate of the animals. The concentration of Zn in the untreated food (142 µg Zn/g dry food) was in the range of concentrations encountered in uncontaminated soils *(33)* and litter *(34).* The concentration of Zn in the metal-dosed food used in our experiment exceeded concentrations encountered in the unpolluted environment, although it was far below the highest values recorded in contaminated litter and soil *(33–36).*

Experimental Setup

Animals were maintained individually in plastic Petri dishes ($2r$ = 9 cm) with perforated side walls. The bottom of the dish was covered with filter paper, wetted in distilled water. Petri dishes with animals were held in glass containers at a temperature of 21°C, a relative humidity of 93%, and under a 16-h light and 8-h dark regime. Ninety animals were submitted to the experiment—half of them fed on nontreated food and the other half fed on Zn-dosed food for 6 d. Food was put on plastic plates and placed in the Petri dishes. On the third day of the experiment, the moldy food was replaced by fresh.

Food consumption was estimated on the basis of the number of fecal pellets. Food consumptions of animals fed on nontreated food and animals fed on Zn-dosed food were compared by the Mann–Whitney test using the SPSS for Windows statistics software. The content of the gut was macroscopically inspected during dissection.

Because it is known that the concentration and metal composition of MTs change in relation to the moult cycle in the blue crab *(Callinectes sapidus) (18,19),* the molting status of every experimental animal was checked. At the beginning of the experiment and before dissection, the sternal deposits, indicating the premolt phase of the molt cycle, were inspected *(37).* Exuviations were recorded every day during the experiment.

Tissue Samples

Cumulative samples of hindguts of each experimental group, fed on nontreated or Zn-dosed food, were prepared for biochemical analysis. Animals were dissected with plastic and glass instruments; hindguts were isolated and longitudinally opened and the content was washed out.

Biochemical Separation

For biochemical separation of cytosolic proteins, a pooled sample of hindgut tissues ($m \cong 100$ mg) of each experimental group was homogenized immediately after dissection with a glass homogenizer and a motor-driven Teflon pestle in 5 mL of ice-cold nitrogen-saturated buffer: 10 m*M* Tris-HCl, pH 8.0, 10 m*M* dithiothreitol (DTT), 0.25 *M* sucrose, 0.1 m*M* phenylmethylsulfonyl fluoride (PMSF). Homogenates were centrifuged at 100,000*g* for 1 h at 4°C (Beckman L8-M ultracentrifuge, bucket rotor SW 65 TI) to obtain postmicrosomal supernatants (cytosol) and pellet. Supernatants were stored at –70°C.

Hindgut postmicrosomal supernatants (2.5 mL) were applied to a Sephadex G-75 (1.7 cm × 60 cm) gel filtration column, previously calibrated with standards of known molecular weights: blue dextran (M_r = 2,000,000) as void volume (V_o), ovalbumin (M_r = 45,000), chymotripsinogen A (M_r = 25,000), myoglobin (M_r = 17,800), cytochrome-*c* (M_r = 12,400), and aprotinin (M_r = 6500) (Serva, Pharmacia). The eluant buffer was 20 m*M* Tris-HCl, pH 8.0, containing 1 m*M* DTT. Special care was taken to avoid oxidation of the metal-binding compounds. Thus, the buffer was previously aerated with N_2. The ultraviolet (UV) absorbance was measured at 280 nm and 220 nm (Perkin-Elmer UV/VIS spectrometer Lambda 11) in 3- to 4-mL fractions and concentrations of total Cu and Zn were analyzed by flame atomic absorption spectrophotometry (FAAS) in an air–acetylene flame with deuterium correction of nonspecific absorption on a Varian AA-5 atomic absorption spectrometer. Detection limits were 5 ng/mL for Zn and 10 ng/mL for Cu. The accuracy of the methods was tested by analyses of two certified reference materials for trace metals:

Table 1
Food Consumption, Estimated from the Number of Fecal Pellets

experimental group	all animals in group Me (Q_1, Q_3)[a]	non-moulting animals[b] Me (Q_1, Q_3)	premoult and moulting animals[c] Me (Q_1, Q_3)
animals fed on untreated food (142 µg Zn/g dry food)	29 (9, 64) n=45	60 (30, 74) n=25	8 (5, 13) n=20
animals fed on zinc-dosed food (793 µg Zn/g dry food)	37 (16, 69) n=45	59 (37, 87) n=27	9 (4, 23) n=18

[a] Me: median, Q_1: 1st quartile, Q_3: 3rd quartile
[b] Animals that did not molt during the experiment were not in the premolt phase.
[c] Animals that were in the premolt phase or molted during the experiment.

DOLT-2 (lyophilized dogfish liver) and TORT-2 (lyophilized lobster hepatopancreas) produced by National Research Council of Canada.

Fractions eluted near the position of cytochrome-*c* (V_e/V_o from 2.04 to 2.13) after gel filtration on Sephadex G-75, which is the expected positon of MT elution, were rechromatographed by size-exclusion chromatography–high-performance liquid chromatography (SEC-HPLC) without prior concentration on a Tosohaas TSK gel G 3000 SW column (60 cm × 7.5 mm), with a Merck Hitachi L-7100 pump. The eluant buffer was 50 m*M* Tris-HCl, pH 8.0, the flow rate was 1 mL/min, and a 200-µL injection loop was used. UV absorbance at 215 nm was measured on-line (Merck Hitachi UV detector L-7400; Hewlet Packard 3390 A integrator) and compared with rabbit liver MT (MT II, Sigma), used as an MT standard.

RESULTS AND DISCUSSION

Food consumption and Molting

Animals (n = 45) fed on untreated food (142 µg Zn/g, 16 µg Cu/g dry food) and animals (n = 45) exposed to Zn-dosed food (793 µg Zn/g, 19 µg Cu/g dry food) for 6 d consumed similar quantities of the food mixture, as estimated on the basis of fecal production (Table 1). According to the Mann–Whitney test, the differences between the two experimental groups were not statistically significant, which is in agreement with previous investigations *(30,31,38).* In both experimental groups, the food consumption of molting and premolt animals was lower than that of the animals

that were not in the molting period during the experiment. This was expected, as animals stop feeding in the premolt phase *(39).*

Thirteen animals molted during the experiment in each of the experimental groups. At the end of the experiment, there were seven and five premolt animals in the group of animals fed on nontreated food and Zn-dosed food, respectively.

Altogether, these results indicate a similar nutritional status, except for Zn supplementation, and a similar molting status of the animals in both experimental groups. Thus, the differences in the cytosolic distribution of Zn and Cu in hindgut cells of *P. scaber,* observed between the two experimental groups, were ascribed to Zn supplementation and not to variability resulting from different food consumption or molting status of the animals.

Distribution of Cu and Zn in the Hindgut Cytosol

After separation of the hindgut postmicrosomic supernatant of Zn-exposed animals by gel filtration on a Sephadex G-75 column, a Cu- and Zn-containing peak was detected in the position V_e/V_o~2, where MTs are expected to elute (Figs. 1 and 2). This peak contained about 12% of total eluted Cu and about 4% of total eluted Zn. In the case of animals fed on nontreated food, no metal-containing peak was detected in this position. SEC-HPLC rechromatography of Sephadex G-75 Cu- and Zn-containing fractions of the Zn-exposed group with V_e/V_o from 2.04 to 2.13 revealed the 215-nm absorbance peak at 21.76 min, coinciding with the absorbance peak of the rabbit MT II standard at 21.13 min (Fig. 3). We consider that these low-molecular-weight Cu- and Zn-binding compounds, detected in the cytosol of the hindgut cells in Zn-exposed *P. scaber,* are Cu,Zn-MT-like proteins. To our knowledge, this is the first report about the characterization of MT-like proteins in isopod crustaceans. Concerning terrestrial invertebrates, MTs have been studied in detail in four groups, namely nematodes, insects, snails, and earthworms *(11).* Association of Zn and Cu with MT-like proteins indicates perturbations in the dynamics of both metals in *P. scaber* exposed to Zn. From studies performed with mammals, it is known that many aspects of Cu–Zn interaction involve MTs. In Zn-supplemented organisms, the induction of MT synthesis in the intestinal mucosa by Zn can be related to decreased Cu absorption, as increased amounts of Cu become associated with mucosal MTs *(40–42).* By analogy, similar processes could be expected to take place in the isopod hindgut. On the other hand, Reeves and Rossow *(43)* and Reeves *(44)* suggest that the effects of high dietary Zn on Cu status are not the result of Zn-induced intestinal MT binding of Cu in Zn-supplemented rats (350 mg Zn/kg of diet, 3 wk) and MT null mice (400 mg Zn/kg of diet, 2 wk), thus preventing its passage into the circulation. Their studies support the explanation that high Zn concentrations affect the expressions or activities of Cu transport proteins *(44,45).* In our study, both the elevation of Cu,Zn-MT-like proteins in the hindgut cytosol and the elevation of Cu concentration in

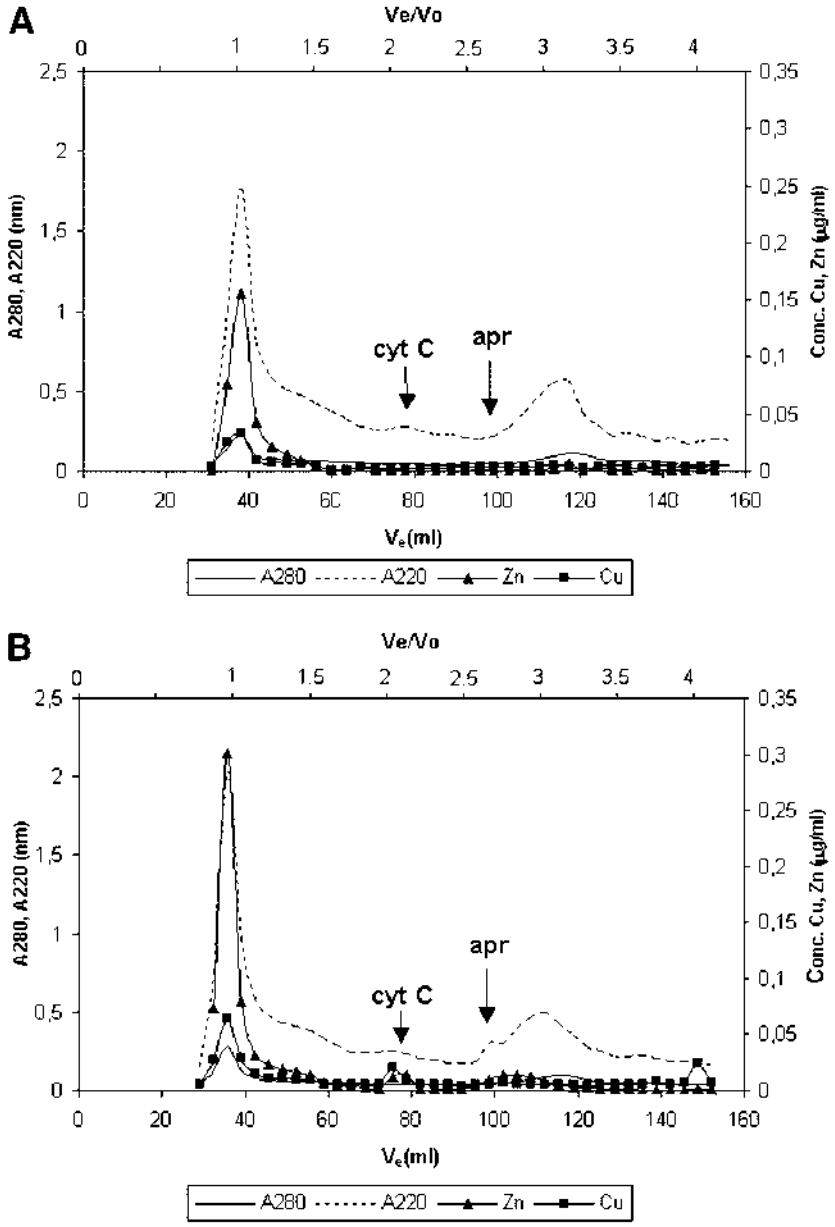

Fig. 1. Sephadex G-75 elution profile of the hindgut postmicrosomic supernatant. Distribution of Cu and Zn in elution fractions and UV absorbances (280 nm, 220 nm) are shown. **(A)** Animals fed on untreated food (142 µg Zn/g dry food); **(B)** animals fed on Zn-dosed food (793 µg Zn/g dry food). cyt C: cytochrome-*c* standard; apr: aprotinin standard.

the hindgut after Zn supplementation were evident. The hindgut pellet of *P. scaber* fed on nontreated and Zn-supplemented food contained 4.3 µg Cu/g wet weight (analyzed by neutron analysis [NAA]) and 6.7 µg Cu/g wet weight, respectively. In the hindgut cytosol, the concentration of Cu

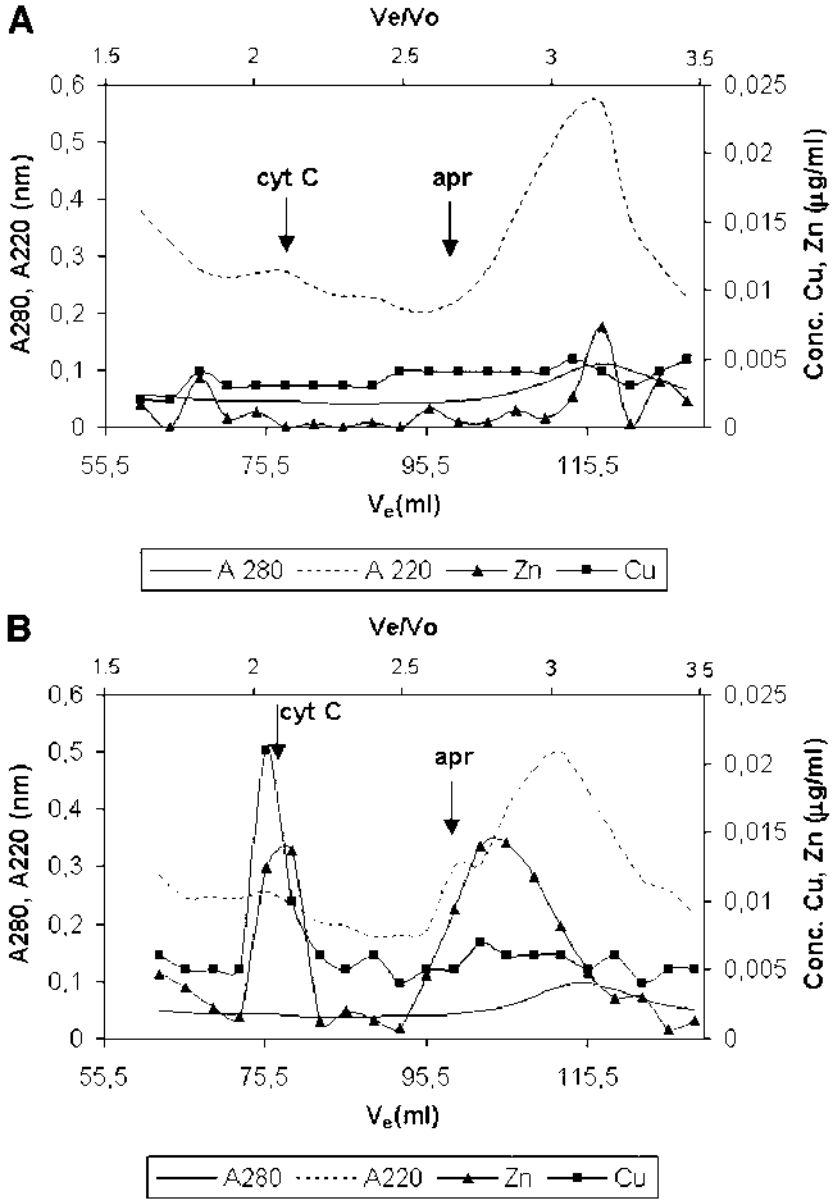

Fig. 2. Partial Sephadex G-75 elution profile of the hindgut postmicrosomic supernatant; V_e = 60 mL to V_e = 125 mL from Fig. 1. Distribution of Cu and Zn in elution fractions and UV absorbances (280 nm, 220 nm) are shown. **(A)** Animals fed on untreated food (142 µg Zn/g dry food); **(B)** animals fed on Zn-dosed food (793 µg Zn/g dry food). cyt C: cytochrome-*c* standard; apr: aprotinin standard.

was elevated in the high-molecular-weight fractions and in the MT-like protein fractions in animals fed on Zn-supplemented food. On the other hand, Cu,Zn-MT-like protein elevation was accompanied by an elevation of Zn concentrations in the high- and very low-molecular-weight fractions

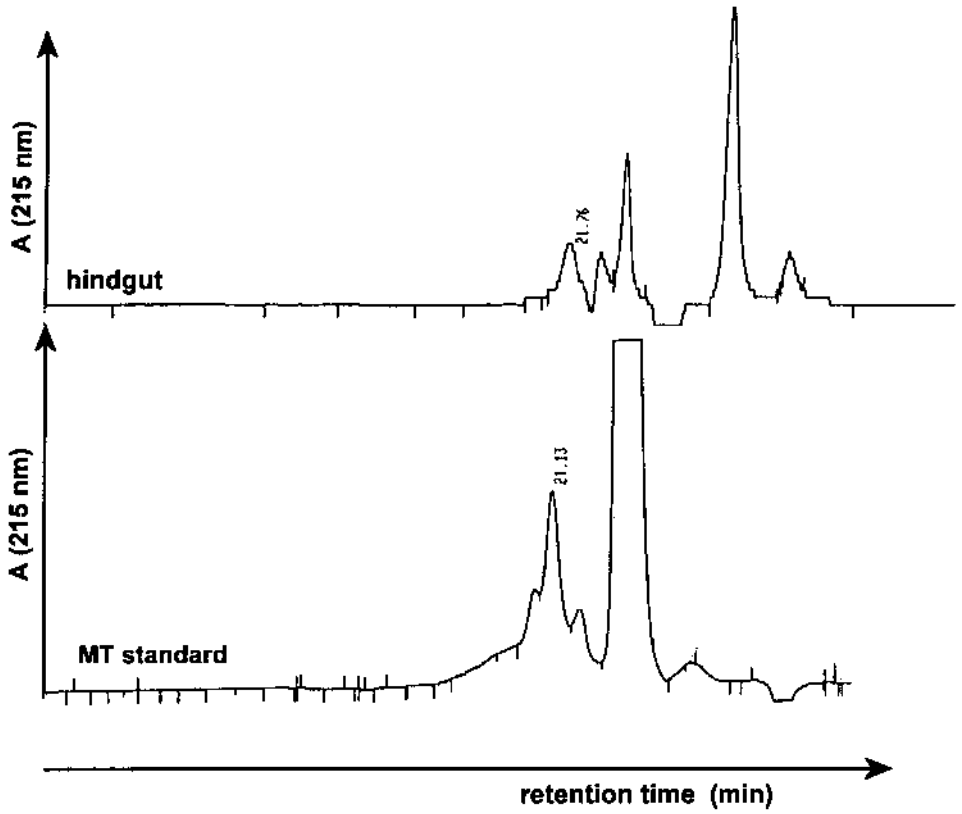

Fig. 3. SEC-HPLC rechromatography of Sephadex G-75 Cu- and Zn-containing fractions with V_e/V_o from 2.04 to 2.13, obtained from the hindgut postmicrosomic supernatant of Zn-exposed animals (793 µg Zn/g dry food). Rechromatography elution pattern of one fraction is displayed. Retention times and UV absorbances at 215 nm are shown. MT standard: rabbit liver MT II standard (Sigma).

of the hindgut cytosol and a decrease in Zn concentration of the hindgut pellet after Zn supplementation. The hindgut pellet of *P. scaber* fed on nontreated and Zn-supplemented food contained 35.9 µg Zn/g wet weight and 26.7 µg Zn/g wet weight, respectively. Together, these results indicate that Zn and Cu dynamics in the hindgut of *P. scaber* are affected at the given Zn supplementation (793 µg Zn/g dry food, 6 d) and that MT-like proteins are involved in this Zn–Cu interaction in *P. scaber* hindgut, which clearly does not rule out the involvement of other mechanisms.

Gel filtration also revealed that in both experimental groups, the major Cu- and Zn-containing peak eluted in the range of the void volume in the Sephadex G-75 chromatograms, representing molecules with M_r > 80,000 (Fig. 1). In Zn-exposed animals, about 73% and 39% of total eluted Zn and Cu, respectively, was present in these fractions. In unexposed animals, these values were 88% and 36% for Zn and Cu, respectively. A comparison

of the highest Zn and Cu concentrations measured in the high-molecular-weight fractions showed that the Zn-to-Cu ratio was about 4.5 : 1 in both experimental groups, but the highest concentrations of both metals in Zn-exposed animals were roughly twice those in unexposed animals. This result also indicates that Zn exposure influences both Zn and Cu dynamics in the hindgut of *P. scaber* and that in addition to MT-like proteins, high-molecular-weight compounds in the hindgut cytosol are also involved.

Gel filtration of the hindgut cytosol of Zn-exposed animals on the Sephadex G-75 column also revealed a very low-molecular-weight Zn-containing peak, which was detected in the position of V_e/V_o~2.8, near the standard aprotinin (M_r = 6500) (Fig. 2). About 10% of total eluted Zn was present in this peak. These very low-molecular-weight Zn-binding molecules were not further analyzed.

In conclusion, (1) Cu,Zn-MT-like proteins were characterized in the hindgut cytosol in the isopod crustacean *P. scaber* fed on Zn-dosed food (793 µg Zn/g dry food, 6 d). To our knowledge, this is the first report about the characterization of MT-like proteins in isopod crustaceans. (2) The elevation of Cu,Zn-MT-like proteins in the hindgut cytosol and the elevation of Cu concentration in the hindgut after Zn supplementation were evident, whereas Zn concentrations were increased in the high- and low-molecular-weight fractions of the hindgut cytosol but were decreased in the hindgut pellet in Zn-supplemented animals. These results indicate that both Zn and Cu dynamics in *P. scaber* hindgut are affected at the given dietary Zn supplementation and that MT-like proteins are involved in this Zn–Cu interaction, which clearly does not rule out the involvement of other mechanisms.

ACKNOWLEDGMENT

The authors thank Dr. P. Zidar for statistical analyses. The investigation was supported by the Ministry of Education, Science and Sport of Slovenia.

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