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DIGESTIVE PROTEASES OF THE LUGWORM (*ARENICOLA MARINA*) INHIBITED BY CU FROM CONTAMINATED SEDIMENTS

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Abstract—We examined potential toxic effects of copper released from contaminated sediments during deposit feeding of the lugworm, *Arenicola marina*. Titration of Cu solution into gut fluids can result in decreases in protease activity if sufficient Cu is added. The effects of Cu on gut proteases were confirmed by incubation of gut fluids with Cu-contaminated harbor sediments. Monitoring of Cu titration into gut fluids shows that enzyme inhibition and quenching of gut protein fluorescence occur only when sufficient Cu has been added to allow inorganic Cu species to become abundant. This threshold level probably represents the exhaustion of strong binding sites that act as protection against enzyme inhibition. Thus, sediments contaminated with Cu may have inhibitory effects on digestive processes in lugworms.

Keywords—Digestive fluids Lugworm Enzyme inhibition Sedimentary copper Deposit feeding

INTRODUCTION

Elevated concentrations of heavy metals, such as Hg, Cu, and Ag, in sediments can be toxic to benthic organisms by inhibiting enzyme systems or growth [1]. The inhibitory actions on enzymes may include conformational changes due to metal binding [2], replacing original metals at catalytic sites [3], and blocking active catalytic sites [3]. In addition, substrates bound by metals may also lead to apparent losses of enzymatic activities [2]. For example, protease may not be able to bind and digest sedimentary substrates (i.e., peptides, proteins) loaded with metals (substrate tanning). On the other hand, metal–enzyme interactions have been successfully used to test the toxicity of sediment and soil samples [4], especially since the introduction of fluorophore-tagged substrates that dramatically enhance the sensitivity of enzyme assays. This method of enzyme assay has proved to be simple, sensitive, reproducible, and correlates well with other biologically based methods [5].

Marine deposit-feeding organisms rely heavily on secreted extracellular enzymes to hydrolyze sediment-bound organic materials. Because of the poor quality of sediment-based food, deposit feeders, such as the lugworm *Arenicola marina*, need to process large amounts of sediments continuously. Such a life style may make their digestive system vulnerable to sediment-bound pollutants. By incubating digestive fluids of two deposit feeders with contaminated sediments (i.e., in vitro digestion), we observed dramatic releases of copper and polycyclic aromatic hydrocarbons (PAHs) from the sediments [5]. The amounts of released pollutants were taken as a measure of their fractions in sediments bioavailable to the digestion of deposit feeders. Although it is operationally defined, the bioavailability of sedimentary Cu is a function of several factors, such as the sediments, species of organisms, and incubation time (i.e., gut retention time) [5]. The neutral gut pH of deposit feeders (Z. Chen and L.M. Mayer, unpublished data) suggests that H^+ is not likely responsible for solubilization of sedimentary metals. An alternative mechanism is complexation of sedimentary Cu by high concentrations of gut amino acids (e.g., up to 1 M) [5] (Z. Chen and L.M. Mayer, unpublished data).

The objective of this study was to determine whether Cu-contaminated sediments causes damage to the digestive system of deposit feeders by monitoring the variation of protease activities in gut fluids under increasing Cu concentrations. We targeted lugworm protease in this study because protease is one of the major extracellular enzymes in the digestive system of the lugworm, and amino acid-based food items in sediments are important nutritional items for deposit feeders [6]. First we simulated the processes of Cu release from contaminated sediments by titration of $Cu(NO_3)_2$ solutions into a composite sample of gut fluid from multiple individuals. Then the pattern of Cu–protease interactions was further confirmed by additional experiments, in which not only protease activities but also protein fluorescence and free cupric ion (Cu^{2+}) activities during the titrations were monitored to probe the mechanisms of protease–Cu interactions. Finally, we examined the effects of Cu released from contaminated sediments on lugworm proteases.

MATERIALS AND METHODS

Lugworms were collected from coastal Maine in summer 1995. The worms were kept in a flowing seawater table for a maximum of 2 d before being dissected for gut fluids. No detectable loss of enzymatic activities was found during this holding time (J. Judd, personal communication). To sample gut fluids, the midgut section was carefully exposed and taken out from a small cut on the body wall, and a pipette tip was used to penetrate and withdraw digestive fluids from this gut section. Digestive fluids from different individuals (normally >10) were pooled in order to achieve enough volume for incubation experiments. Particulate materials in gut fluids were removed by centrifugation at 8,000 *g* for 30 min. The gut fluids then were stored in plastic tubes at −80°C until experiments.

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Sandy, oxic sediments (surface 5 mm) were collected at intertidal zones in Boothbay Harbor (BH), Maine (total [Cu] ≈ 1,113 ppm at BH site) and two stations in Portsmouth Harbor (PH), New Hampshire (total [Cu] ≈ 182 ppm and 204 ppm for sites PH-1 and PH-2, respectively). Excess copper in sediments presumably originated from nearby shipyard activities. Analysis of various metals (Cu, Cd, Pb, As, Cr, Se, Ni) in sediments indicate that Cu and Pb are the dominant contaminants, although an earlier report showed that the site at Boothbay Harbor was also contaminated with Sn and Zn [7], and sediments from Portsmouth Harbor have been contaminated with Hg (F. Short, personal communication). The sediments were stored in a refrigerator at 4°C for a week until used in experiments. At least one of the sediments (Boothbay Harbor site) was lethal to lugworms as shown by maintaining lugworms in the sediment. During a 48-h period, 100% of worms (five individuals) died, in contrast to 100% survival in control sediments from the worms' native habitat. This short-term lethal effect suggests death by factors other than starvation.

Experimental procedure

Incubation of gut fluids with polluted sediments and analysis of metals in gut fluids were done according to Mayer et al. [5]. Briefly, three replicates of wet sediments were incubated with gut fluids at a ratio of about 1 g wet weight to 2 ml fluid in plastic centrifuge tubes for 240 min at room temperature. Incubation was stopped by centrifuging the mixture at 8,000 *g* for 30 min, and the supernatant fraction was used for measurements of metals and protease activity. Kinetic experiments were done by varying the incubation time from 2 to 360 min. Control experiments included gut fluids without sediments, seawater with sediments, and gut fluids with sediments from the worm's habitat. Metals in the gut fluids and seawater were measured on a graphite furnace atomic absorption spectrophotometer (5100ZL, Perkin Elmer, Norwalk, CT, USA).

Concentrations of total dissolved amino acids (TAA) were analyzed according to Mayer et al. [8]. Briefly, samples were first hydrolyzed by 6 N HCl on a hot bath (110°C) for 22 h, and the HCl-hydrolyzed samples were then derivatized with orthophthaldialdehyde followed by fluorometric detection.

Free Cu^{2+} activity and pH during Cu titrations were measured in a glass titration cell by a Cu-ISE (ion selective electrode, Orion 9629, Orion, Beverly, MA, USA) and a pH electrode (Orion 911600) connected to an Orion EA940 ion analyzer at 22 ± 1°C. MOPS (4-morpholinepropanesulfonic acid) buffer (i.e., 0.01 M MOPS and 0.01 M $NaNO_3$, pH 7) was used throughout the experiments in this study. Cupric ion standards were freshly prepared every day with 0.1 M $Cu(NO_3)_2$ stock (Orion 942906) and deionized water (Milli-RO/Milli-Q system, Millipore, Bedford, MA, USA). A stream of nitrogen gas was purged through the titration cell to prevent oxygen interference during the measurement. Photoreaction on the Cu-ISE was minimized by keeping the titration apparatus in a dark room. Before each titration experiment, the Cu-ISE was equilibrated for 120 min with the initial titration solution containing MOPS buffer and digestive fluids (see the next paragraph), in order to reach a steady voltage reading (drift $< \pm 0.2$ mV). The equilibrium time can be shortened considerably to 2 to 15 min once addition of $Cu(NO_3)_2$ is started, as increasing Cu concentration requires shorter equilibrium time. The Cu-ISE was calibrated against cupric ion buffers [9] that were made of various amounts of Cu in histidine or glycine (10^{-3} or 10^{-4} M) solutions based on MOPS buffer. In all cases, the slope of the calibration curve was not significantly different from the theoretical 29.3 mV per decade expected at 22°C. A chemical equilibrium program, MINEQL+, was used to calculate free Cu^{2+} activities in the buffers. This technique allows measurement of Cu^{2+} activity in the range 10^{-15}–10^{-5} M.

To start Cu titration experiments, a small volume of $Cu(NO_3)_2$ solution was spiked into the titration cell containing 50 ml of MOPS buffer with 1% gut fluid (i.e., 100× dilution). After the recording of Cu^{2+} activity and pH at each time, an aliquot of the solution (100 μl) was withdrawn from the titration cell and further diluted 10× with MOPS buffer (i.e, 1,000× dilution) to use for protease assay.

Protease activity in gut fluids (two replicates per sample) was measured by monitoring the initial hydrolytic rates of substrate AMCA (L-alanine 7-amido-4-methylcoumarin) on a fluorescence spectrophotometer (F4500, Hitachi, Tokyo, Japan). This method has been described elsewhere [8] but modified here by use of MOPS buffer, because MOPS has negligible interactions with cupric ion at the experimental ionic strength (unpublished data). In the substrate AMCA, alanine is attached to MCA (methylcoumarinyl amide) via a peptide bond that upon hydrolysis produces alanine and MCA. The nonspecific protease activities measured by using AMCA are comparable with those based on other substrates [8]. However, the structure of this substrate mimics peptides with short-chain neutral amino acids, thus limiting the types of proteolytic enzymes being monitored. Of the five serine proteases isolated in lugworm gut fluids [10], this substrate can only be used to detect the elastase-like proteases [11]. Nevertheless, the measured protease activity represents a major species of protease in gut fluids [10] and thus serves as a useful indicator of the digestive functioning of the lugworm.

Experiments studying Cu quenching of fluorescence of gut proteins and AMCA and its hydrolyzed product MCA were performed in a glass cuvette with the same media as in the protease assay. The gut fluids were first diluted 1,000× (setting [TAA] at about 10^{-4} M), while the concentrations of AMCA or MCA were about 10^{-6} to 10^{-5} M. To monitor the AMCA quenching curve, the proteases in gut fluids were deactivated by microwave twice to boiling point before mixing with substrate AMCA, so no AMCA hydrolysis occurred during Cu titration. After titrating cupric ion into the system, fluorescence emission intensity was monitored on a fluorescence spectrophotometer (F4500, Hitachi, Tokyo, Japan) at excitation/emission wavelengths of 280/340 nm for proteins (the tryptophan peak), 325/390 nm for AMCA, and 340/445 nm for MCA.

RESULTS AND DISCUSSION

High concentrations of copper leads to inhibition of proteases

Titration of $Cu(NO_3)_2$ into the composite gut fluids and those of two individual lugworms (W17 and W39) showed that Cu concentrations of $2.5–10 \times 10^{-3}$ M can inhibit protease activities in gut fluids (Fig. 1). Protease activities decreased sharply above this break-off point (BOP). The variation in Cu concentrations is explained by coincident variation in TAA, so that the BOP always occurred at a TAA : Cu ratio of about 50 (Fig. 1). The control experiment without Cu addition showed no apparent loss of protease activities during a 9.5-h period at room temperature, indicating that the enzyme inhibition above the BOP was due to the addition of Cu to the system.

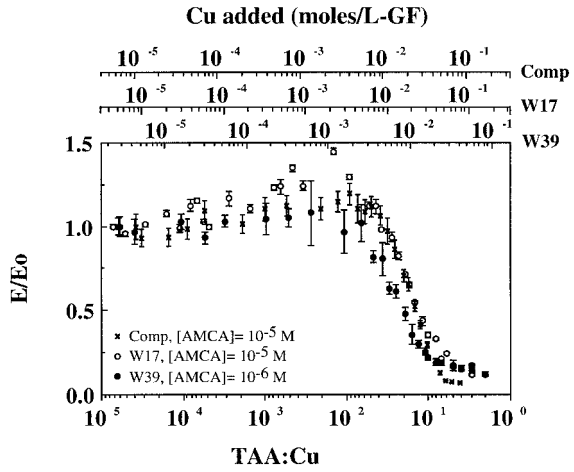

Fig. 1. Dose–response curves of protease activities in three gut fluids during Cu titration. The protease activities are expressed as a non-dimensional parameter (E/Eo), which is the fraction of their initial values. TAA : Cu ratios were calculated based on the assumption that there are no losses of gut amino acids throughout the titration. Total Cu concentrations were calculated as moles Cu per liter gut fluids. *Y*-error and *X*-error bars represent ±1 SD (including analytical errors and their propagation) of the data. Some error bars, especially *X*-error bars, are smaller than the size of the data points. The initial protease activity in the composite gut fluid (∗) was 648 μM/min (=μmole AMCA hydrolyzed per liter gut fluid per min), while those of W17 (○) and W39 (●) were 188 and 46 μM/min, respectively.

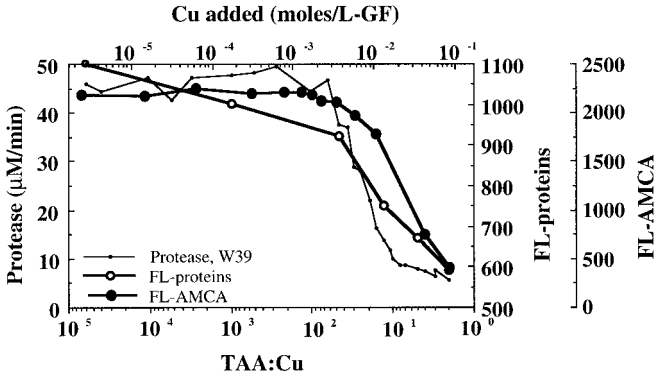

Fig. 2. Gut TAA and substrate AMCA fluorescence quenched by Cu^{2+} titration. The gut TAA quenching curve was recorded during Cu^{2+} titration of gut fluid W39. The excitation/emission wavelength for gut TAA was 280/340 nm, while that of AMCA was 325/390 nm. Units: Cu added (M) = moles of Cu per liter of gut fluids.

This BOP behavior suggests that gut proteases were able to tolerate increasing Cu concentrations up to a certain extent. This threshold effect, in addition to the role of gut amino acids (AA) as Cu-binding ligands (unpublished data), implies that gut AA may serve as a Cu buffer against the inhibition of proteases, probably due to preferential binding of Cu to catalytically irrelevant sites in AA of the gut fluids. The variation in the BOPs between the three experiments (Fig. 1) is small in comparison to the wide range of TAA : Cu ratios, although TAA concentrations of the two gut fluids differ by about threefold. AMCA has little effect on the TAA : Cu ratios in the experiments, as its concentrations are much lower than the gut TAA on the order of 10^{-4} M (after 1,000× dilution) during the protease assay (Fig. 1). Lowering of AMCA concentration to 10^{-6} M in the W39 titration does not result in significant change in the TAA : Cu ratio of the BOP.

Although protease activities remained unchanged over a wide range of initial Cu concentrations, a slight rise occurred in two of the experiments (W17 and Comp) with the addition of Cu, before falling sharply at the BOP. We failed to see a similar rise in the other gut fluid (W39), possibly due to its low initial activity and high standard deviations (Fig. 1). The increases in protease activity before the BOP in sample W17 and Comp suggest that the enzymes may be optimized within a certain range of Cu concentrations, which is analogous in some respects to pH optima (concentrations of proton) [12].

Mechanisms of Cu inhibition

Cu interactions with gut AA and substrates were probed by monitoring the quenching of fluorescence signals of gut AA, AMCA, and its hydrolysis product MCA during titrations of Cu. AA fluorescence of W39 quenched slowly during the initial 2.5×10^{-3} M addition of Cu^{2+} (TAA : Cu > 60), followed by a faster decline beyond the BOP (Fig. 2, similar in W17, data not shown). Significant attenuation of AMCA fluorescence, however, began only above the BOP of protease activity. These results suggest that Cu preferentially binds to gut AA at higher TAA : Cu ratios (i.e., on the plateau) until the buffering pool of gut ligands is saturated. This preferential binding is confirmed by comparing the fluorescence quenching curves of AMCA with and without gut AA of W39 (Fig. 3); quenching of AMCA occurs at lower Cu concentrations in the absence of gut AA. On the other hand, the presence of AMCA does not affect the AA fluorescence quenching curve (data not shown), corroborating a weak interaction of Cu with AMCA in comparison to that with gut AA.

The nature of the quenching, however, has important implications for the inhibitory mechanisms of Cu on proteases, because fluorescence quenching can be caused by collision between Cu and excited fluorophores (dynamic quenching) or by binding of Cu to fluorophores at ground states (static quenching) [13]. The fact that Cu does not quench MCA at the experimental pH (data not shown), in contrast with its strong quenching of AMCA (Fig. 3), suggests that the alanine group in AMCA plays an important role in quenching. Dynamic quenching cannot explain this differential behavior between AMCA and MCA, as it is unlikely that the slightly larger sized AMCA collides with Cu much more efficiently than

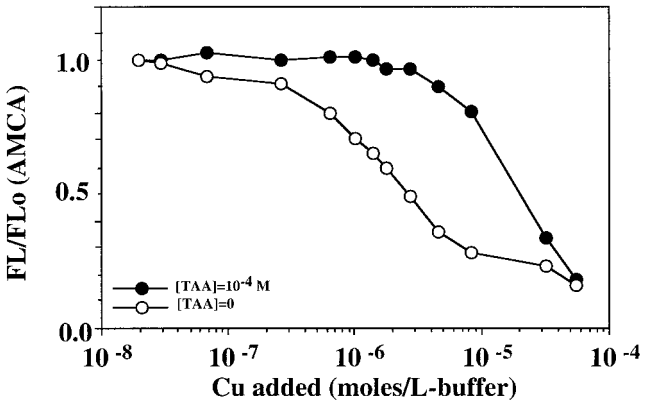

Fig. 3. Fluorescence quenching curves of AMCA with (●) and without (○) the buffering effect of gut TAA. The quenching effect is expressed as fractions of fluorescence without Cu addition at excitation/emission wavelength of 325/390. The titration media include 2 ml pH 7 MOPS buffer, 10^{-6} M AMCA, with or without 10^{-4} M TAA in W39. The amount of Cu added was expressed as moles Cu per liter of the titration media.

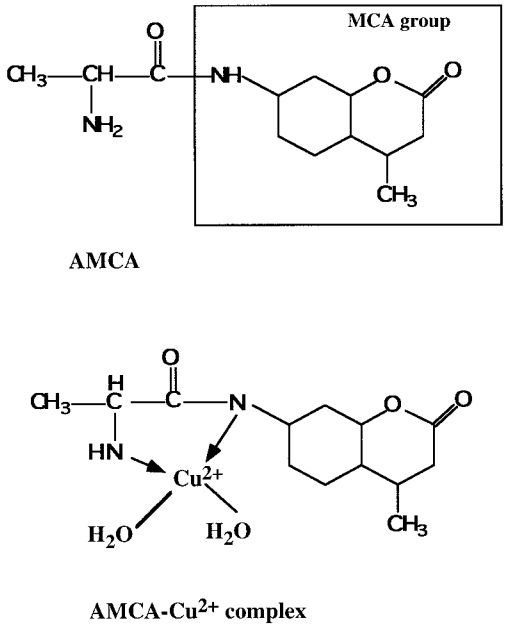

Fig. 4. The structure of AMCA, MCA, and proposed model for the Cu^{2+}–AMCA complex. One mole AMCA could be hydrolyzed into 1 mole alanine and MCA, respectively, by lugworm proteases. The Cu–AMCA complex may involve an amide-N and an amino-N from the AMCA and two water molecules.

MCA at the same temperature. Difference in structures (Fig. 4), however, suggest that insufficient binding sites prevent the formation of a stable complex between Cu and MCA, while the addition of alanine allows the formation of a bidentate Cu complex involving the amino-N and amide-N in AMCA (Fig. 4). This analysis suggests that quenching of AMCA by Cu is likely due to complexation.

The mechanism of Cu quenching of AMCA fluorescence could serve as a model for the interaction between Cu and proteases. Free Cu^{2+} activities measured by the Cu-ISE dramatically increased at the BOP of proteases (Fig. 5), consistent with the simultaneous losses of protease activity and fluorescence (Fig. 2). A sharp increase in Cu^{2+} activities during titration was often observed around TAA : Cu of 50–100 (unpublished data). This result suggests that Cu becomes a potent inhibitor/quencher only after the availability of free Cu^{2+}

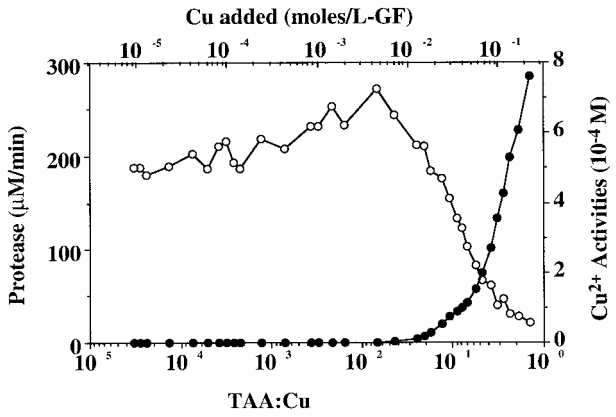

Fig. 5. Protease activity (○) and free Cu^{2+} activity (●) versus total Cu and TAA : Cu for titration of gut fluid W17. Protease activity (μM/min) = μmole AMCA hydrolyzed per liter gut fluid per min; Cu added (M) = moles Cu per liter gut fluid.

reaches a certain level (e.g., BOP), which is clearly controlled by the amount and type of ligands in the system. However, the involvement of other inorganic Cu species, besides free Cu^{2+}, in inhibition/quenching should not be ruled out, as the activity of other inorganic species (e.g., $CuOH^{+}$) also increases along with that of free Cu^{2+}. Working on a Cu-fulvic acid system, Sarr and Weber [13] found that at pH >5, bound Cu dramatically quenches fulvic acid fluorescence (i.e., static quenching); unbound metal ions, however, do not quench the fluorescence (i.e., no dynamic quenching). These lines of evidence suggest that quenching of AA fluorescence, and presumably inhibition of proteases in our experimental system, are probably also due to Cu binding.

The toxicity of Cu could be repressed at high TAA : Cu ratios (>BOP) due to its preferential complexation with stronger binding sites in gut AA that do not affect catalytic function. Lugworm gut fluids contain a suite of binding sites with a spectrum of conditional Cu binding constants, log K' = 5.5–12.5 (unpublished data). We have found the bulk of Cu solubilization by gut AA to result from binding to histidine residues in digestive fluids (unpublished data), which account for 2 to 5% of the TAA in lugworm gut fluids [8]. These sites comprise not only enzymes but also amino acid-based food items from sediments [8]. The total number of these strong binding sites, thus, may determine the critical ratio of TAA : Cu (BOP) at which the inhibitory effect sets in. Apparently, these strong binding sites complex with Cu with no impact on protease activity and relatively little impact on fluorescence. It has been well documented that strong metal binding ligands, such as EDTA [14] and histidine [15], prevent enzymes from being deactivated by metals. This result suggests that Cu-complexation-induced conformational change in proteases at both above and below BOP, in addition to Cu binding at the catalytically active site at total [Cu] > BOP, could explain inhibition.

The losses of protease activity could partially be due to the formation of Cu–AMCA complex (substrate tanning), in addition to inhibition of proteases. The decline of AMCA fluorescence (Fig. 2) above the BOP was apparently due to complexation to AMCA, which we assume to be linearly proportional to fluorescence losses. We also assume that free AMCA is the only available species of protease substrate (i.e., Cu–AMCA is unavailable). Because protease activity correlates linearly with substrate concentrations (data not shown), we can estimate the corresponding loss of protease activity (Fig. 2) due to decreasing AMCA activity. Our calculation indicates that the decreasing AMCA activity could only account for a small portion of the losses in protease activity during most of the titration processes, except at the end of the experiment (i.e., TAA : Cu ≤ 6). Therefore, we conclude that protease inhibition during Cu titration resulted both from Cu–AMCA and Cu–protease interactions, but Cu–protease interaction was responsible for most of the inhibitory effects. Nevertheless, other mechanisms, such as damage by Cu-induced hydroxyl radicals [15], may also contribute to the observed protease deactivation in this study.

Digestive proteases inhibited during in vitro digestion

Consistent with our early findings [5], lugworm digestive fluids dramatically released Cu from contaminated sediments (Table 1), about two to three orders of magnitude more Cu than in the seawater incubation control (data not shown). There were minor releases of Pb, Cr, and Zn from these contaminated

Table 1. Metal concentrations (μM) in gut fluids of lugworm before and after incubation with sediments (separated by →)[a]

	Experiments	PH-1	PH-2	BH
Cu	A	10.3 → 86	10.3 → 172	10.3 → 561
	B	9.8 → 48–144	10.6 → 55–134	10.0 → 308–829
	C	4.0 → 216	4.0 → 150	4.0 → 1,601–4,396
	D	—	—	7.2 → 96–388
	E	—	—	11.2 → 605
As	A	30 → 24	30 → 25	30 → 22
	B	35 → 27–31	31 → 24–28	32 → 23–27
	C	28 → 25	28 → 25	28 → 15–24
	D	—	—	61 → 50–56
Pb	A	0.07 → 0.18	0.07 → 0.37	0.07 → 1.27
	B	0.35 → 0.36–0.40	0.23 → 0.43–0.54	0.31 → 0.74–1.39
	C	0.06 → 0.45	0.06 → 0.56	0.06 → 0.38–1.94
	D	—	—	0.00 → 0.48–0.97
	E	—	—	0.09 → 0.38
Cr	A	0.15 → 0.51	0.15 → 0.33	0.15 → 0.34
Se	A	1.6 → 1.3	1.6 → 1.4	1.6 → 1.1
Cd	A	0.09 → 0.19	0.09 → 0.14	0.09 → 0.09
	B	0.07 → 0.12–0.23	0.07 → 0.11–0.25	0.14 → 0.04–0.07
	C	0.11 → 0.20	0.11 → 0.14	0.11 → 0.11–0.14
	D	—	—	0.12 → 0.11
	E	—	—	0.09 → 0.07
Ni	D	—	—	17 → 15–17
	E	—	—	23 → 19
Zn	E	—	—	110 → 136

[a] Sediments used in experiments A–E were collected at the same location, but at two different times. The range of the data within each experiment was due to differences in incubation time (i.e., kinetic data). Difference in sediments and gut AA among experiments may lead to variation in metal bioavailabilities; — indicates no data.

sediments, while none of the other metals monitored (Cd, As, Se, Ni) had significant release (Table 1). Incubation of gut fluids with the sediment from the worm's habitat resulted in negligible metal release (data not shown). The amount of Cu mobilized by gut fluids varied according to the types of the sediments and incubation time. For a given batch of digestive fluids, sediments from the Boothbay site released more Cu than those from the Portsmouth sites, suggesting a larger bioavailable pool in the BH sediment. Longer incubation times generally resulted in more Cu mobilization by gut fluids, and the kinetics of this process will be addressed in a separate paper.

Protease activities versus TAA : Cu ratios (Fig. 6) showed a similar trend as in the titration experiments, except that the BOP occurred at a TAA : Cu ratio of 200 to 300, in comparison with 50 in the former experiments (Fig. 1). The higher BOP in sediment incubation experiments may be due to factors such as other unidentified enzyme inhibitors in the sediments or preferential involvement of digestive proteases in Cu solubilization from sediment grain surfaces. Nevertheless, the similarity in BOPs suggest that Cu was the primary protease inhibitor in these sediments.

The inhibition of protease suggests that the digestive system of lugworms, and perhaps those of other deposit feeders, are vulnerable to Cu-contaminated sediments. Besides attacking the respiratory and reproductive systems of deposit feeders, bioavailable Cu in sediments may cause damage to the digestive system and contribute to an overall lethal effect. On the other hand, the invariant protease activities at TAA : Cu of >200 suggests that gut proteases are able to tolerate the increasing Cu concentrations to a certain extent. The impact of sediment-bound metals to the digestive physiology of lugworms, and deposit feeders in general, thus deserves further study.

Proteases, as well as the other enzyme systems proposed earlier [1,4], might serve as a screening bioindicator for sediment toxicity. However, this study indicates that the total Cu concentration in gut fluids is not a good indicator of toxin dose, because of the large variation in Cu concentrations at the BOP among the sediment incubation (1×10^{-3} M, Fig. 6) and the titration experiments ($2.5–10 \times 10^{-3}$ M, Fig. 1). The

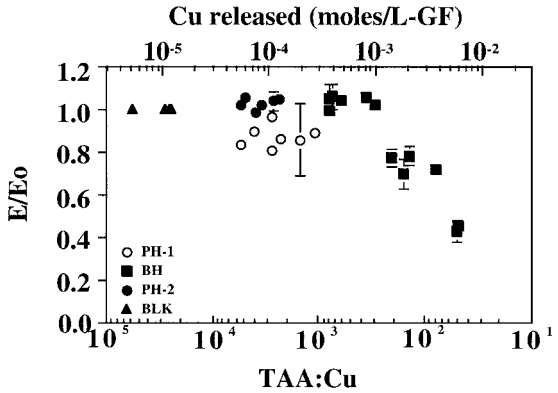

Fig. 6. Protease activities in lugworm gut fluids versus Cu released from contaminated sediments and TAA : Cu ratios. ○ = PH-1, ● = PH-2, ■ = BH, and ▲ = gut fluids before incubation with sediments (BLK). Protease activities were expressed as fractions of their initial values. Cu released (M) = moles of Cu per liter of gut fluids. *X*- and *Y*-error bars are ±1 SD of the data. Some error bars, especially *X*-error bars, are smaller than the size of the data points. Variations among the data of the same sediments resulted from difference in incubation time (i.e., kinetic data).

TAA : Cu ratios in the system, or activity of certain inorganic metal species (e.g., free Cu^{2+}, Fig. 5), may be more useful master variables in studying metal-related toxicity.

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