Studies on intake of heavy metals by *Bradybaena similaris*, land snails, by XAFS measurement

Mitsuko Yasoshima*, Motoyuki Matsuo, Akihito Kuno, Bokuichiro Takano

Department of General Systems Studies, Graduate School of Arts and Sciences, University of Tokyo, 3-8-1 Komaba, Meguro-ku, Tokyo 153-8902, Japan.
E-mail: mikkoyao@dolphin.c.u-tokyo.ac.jp

We have applied XAFS in order to determine both the chemical form and the place where heavy metals are stored in cultivated land snails. From Cu and Zn XANES spectra, the shells showed similar patterns as those of soft tissues and not like carbonates. This indicates that heavy metals are not completely taken into carbonate structures but are present within organic components in the shells. In addition, Cu XANES spectra of the samples showed low absorption edge-energy in the order of hepatopancreas, mantle, body, and shell. By comparing samples with standard reagents, each of which has only S- or O-ligand, it was found that the metals in hepatopancreas exist mostly as S-bound chemical components. To quantify the relative abundance of S-bound chemical component, partial least-squares (PLS) regression was applied. The PLS result indicated that for Cu, S-bound compound was higher in the order of hepatopancreas > mantle > body >shell.

Key words: Land snail, heavy metal, bioaccumulation, XANES, PLS

1. Introduction

The mechanism in which the animals accumulate heavy metals is important to understand the cycling of heavy metals in the environment. Studies on terrestrial mollusks, particularly snails, suggest that variation in metal accumulation in their soft tissues is due to the body size, age, and season (Beeby & Eaves, 1983). In our previous studies, we have seen growth differences among snails given diets supplemented with various levels of heavy metals (Yasoshima & Takano, 2000). Owing to the snails' complex metabolism, the details of a complete biochemical relationship between the metal concentration in their environment and their tissues are not well known. Previous studies have demonstrated that heavy metal elements, once accumulated into mollusk soft tissues, may decrease when the mollusk is moved to a noncontaminated environment (Williamson, 1980; Beeby & Eaves, 1983).

The advantage of the use of mollusks is that the metabolic elimination of metals accumulated into their soft tissues is relatively slow compared with other organisms (Cossa, 1989). On the other hand, the shells are known to preserve the accumulated metals within their crystalline, calcitic or aragonitic structure, and the metals are not lost even after the death of the organism (Watson *et al*., 1995). If they are firmly stored within their bodies, it is most probable that the metals exist in complexes with ligands of S, O, or N. Dallinger *et al*. (1989) stated that the snails' high capacity for metal accumulation and storage of metals is attributed to the induction of metal-binding proteins belonging to the metallothioneins. Elucidating how the organisms store these metals is the primary goal of this research.

In this study, mainly Cu and Zn in the shells and the body parts of *Bradybaena similaris*, land snail, were investigated using XANES technique. XANES spectroscopy, unlike biochemical analysis and serial chemical extraction, has an advantage as an *in situ* chemical speciation technique, due to its nondestructiveness. In addition, an attempt was made to quantify the chemical species of each heavy metal by applying partial least-squares (PLS) method, which can deconvolute overlapped XANES spectra of mixtures (Kuno *et al*., 1999; Kuno & Matsuo, 2000).

2. Materials and Methods

2.1 Sample Preparation

B. similaris samples were cultivated within an incubator, to eliminate metal contamination other than the targetted metal elements: Cu and Zn. *B. similaris* was chosen, for the reasons that they are easily found and are widely spread throughout natural environment in Japan, and also because they are relatively easily cultivated within laboratories. The parent snails were collected from Metropolitan area of Tokyo, Japan. In order to reflect the randomness of snails in a natural habitat, and in size, 20 adult snails were paired to make a mass of 1000 juvenile snails. Then the snails were randomly selected into 10 groups, 40 snails in each. The snails were cultivated separately in small acrylic boxes, 100×60×20mm, until the fourth week, followed by cultivation in the boxes twice the size of the first.

The cultivation was run for a total of 12 weeks at 25ºC under light control of 18L:6D. They were given artificial diets containing Cu and Zn. Cu and Zn were chosen to represent essential elements. The snails were weighed once every week to observe the influences of the rearing environment on snail growth. After 12 weeks of cultivation, the shells of the snail were separated from their bodies. Then, the bodies were dissected into three parts: mantle, hepatopancreas, and the rest of the body. The shell was carefully washed by an ultrasonicator in ultra-clean deionized water prepared with a Millipore Elix-3, vacuum-dried, and powdered in an agate mortar with an agate pestle. The body parts were likewise dried and powdered. The powdered samples were then packed in oxygen-impenetrable bags.

2.2 XAFS experiment

To the samples prepared above, XAFS technique was applied to investigate the chemical species of Cu and Zn in the bodies and shells of the snails. Cu and Zn K-absorption spectra of the samples were measured in the fluorescence mode using a Si (111) two-crystal monochromator and a Lytle-type detector (Lytle *et al.*, 1984) at BL-9A of KEK Photon Factory, Japan. The standard reagents were measured as well to compare with the samples, and for PLS modelling.

The standard chemical reagents, whose ligands are S, N, and/or O were chosen to quantify the relative abundance of the metals. As S-bound compounds, Cu-diethyldithiocarbamate (Cu-S) and Zn-diethyldithiocarbamate (Zn-S) were chosen. As organic O-ligand compounds, Cu-acetate (Cu-Oo) and Zn-acetate (Zn-Oo) were used. As N/O-bound compounds, Cu-EDTA (Cu-NO)was used. Furthermore, Cu-carbonate (Cu-Oi) and Zn-carbonate (Zn-SOi) were chosen as inorganic O-bound compounds.

These reagents were paired and powdered to make a series of standard pairs composed of different chemical composition percentage: Cu-S/NO (Cu-S and Cu-NO), Cu-SOo (Cu-S and Cu-Oo), Cu-SOi (Cu-S and Cu-Oi), Zn-SOo (Zn-S and Zn-Oo), and Zn-SOi (Zn-S and Zn-Oi). The standard reagents were diluted in BN powder and made into pellets, in a diameter of 10mm, and

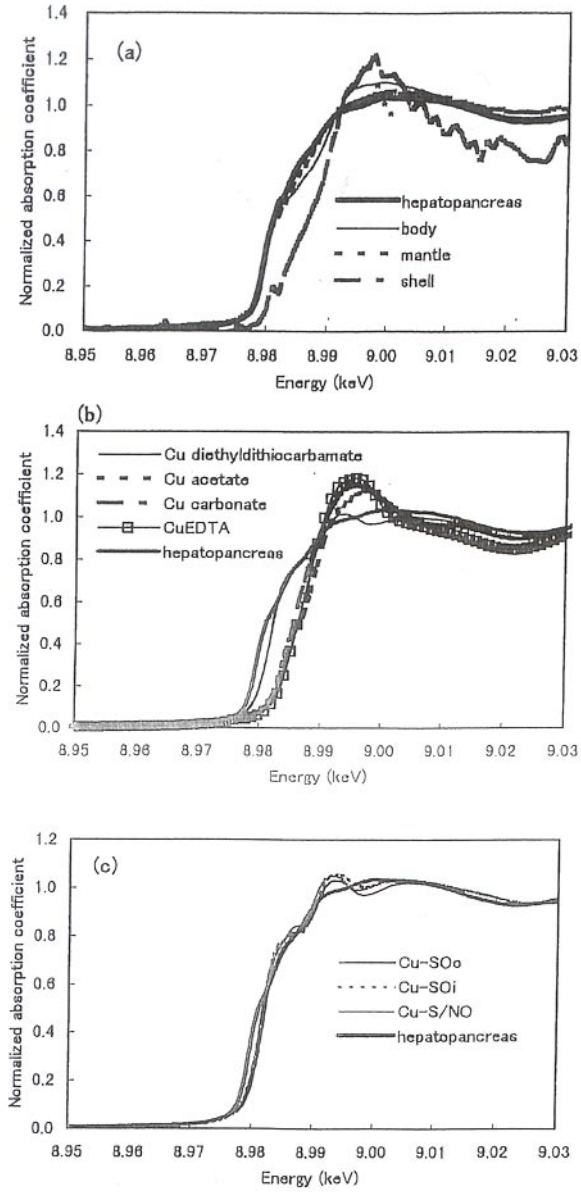

Figure 1
(a)Cu K-absorption of the samples, (b) Cu K-absorption of standard reagents and hepatopancreas, (c) Cu K-absorption of best fitted curve and hepatopancreas

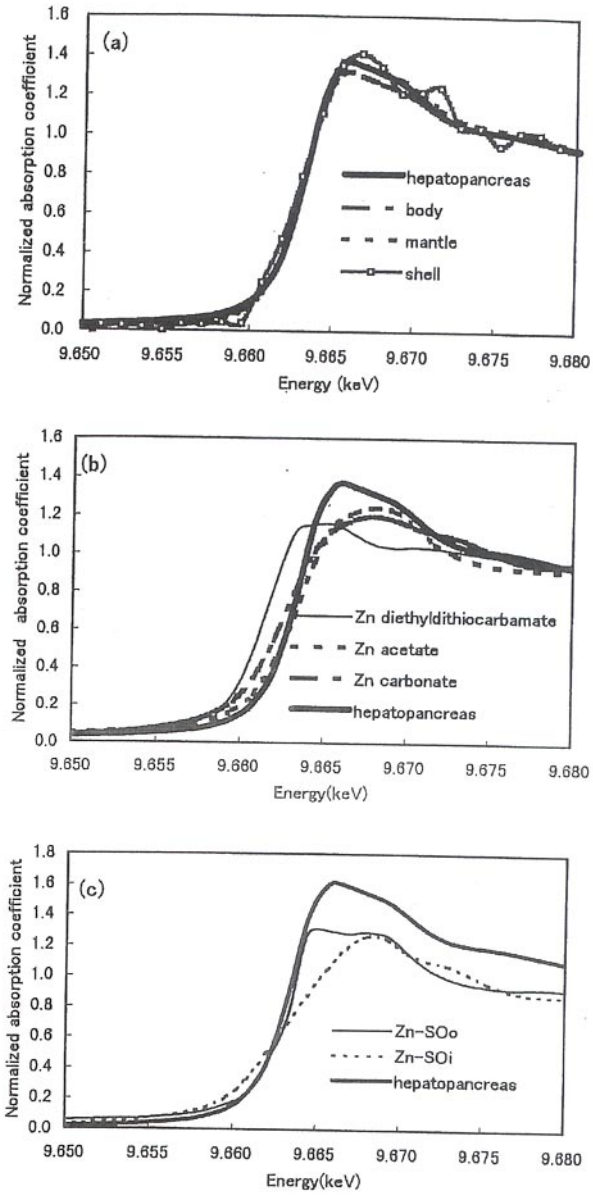

Figure 2
(a)Zn K-absorption of the samples, (b)Zn K-absorption of standard reagents and hepatopancreas, (c)Zn K-absorption of best fitted curve and hepatopancreas.

sealed into anaerobic pack. These series of standard reagents were applied for PLS modelling.

2.3 PLS

PLS was applied for the series of mixed standard reagents in the calibration sets, after the following procedures were operated: subtraction of background, normalization by edge step, and extraction of XANES spectra at Cu-K and Zn-K absorption. PLS models were constructed using these spectrum data as body by PLS modelling of 150 independent variables for Cu. There are 150 points in Cu XANES spectrum. For Zn, 128 independent variables were used to construct PLS model; there are 128 points in Zn XANES spectrum. The relative abundance of the standard reagents, the pairs listed above, was used as dependent variables. The normalized XANES spectral data and the relative abundances ranging between 0 and 1 were subjected to the PLS algorithm (Lindberg *et al.*, 1983).

3. Results and Discussion

The Cu K-absorption energy edge of the snail sample spectra appeared, from lower energy, in the order of body, hepatopancreas, mantle, and shell (Fig.1 (a)). From our previous report (Yasoshima & Takano, 2000), the concentrations of Cu in these parts were in the order of body, hepatopancreas, mantle, and shell. The body includes every organ besides the above-mentioned organs; therefore, it appeared higher in Cu

concentration. This order of spectra, besides the body, corresponds to the relative abundance of chemical forms of this metal within parts of the snails. This suggests a presence of possible metabolism involving cystein-rich protein, as mentioned by Dallinger *et al*. (1989). Such protein stores Cu particularly within the hepatopancreas, and the metal detoxifying metabolism may shift the absorption edge slightly towards lower energy. The hepatopancreas spectrum was compared with those of standard reagents as shown in Fig.1 (b). It was observed that at the K-absorption edge, the Cu spectrum appeared closer to the S-bound reagent.

Then, PLS regression was applied to quantify the difference observed in the spectra of the parts of the snails. By applying the PLS method to the mixtures of standard compound series, the relative abundance of each compound was estimated. The curve-fitted spectra, resulting from PLS modelling were compared with that of hepatopancreas in Fig. 1(c). The residual in the fits of spectra provides a criterion for deciding which standard compound series fits the samples best (Lindberg *et al.*, 1983). In Table 1, the residual values are shown, along with the estimated content, in %, of S-bound and O-bound Cu species in each part of the snail bodies. The smaller the residual values created a better fit. When negative values appear in O-bound result, it indicates that the actual spectra appear in the lower energy region than the pure S-bound reagent. It may show that Cu-SOi is inadequate for the snail's soft tissues as the standard reagent series.

Considering the possibilities of the presence of such reagents, we have concluded that from the ratios of S-bound and O-bound species within each part of the snail bodies, the chemical species were completely different among the body parts and the shell. In the hepatopancreas, the metal is said to be stored and detoxified, particularly by the presence of metallothionein (Nordberg & Kojima, 1979). In addition, the Cu PLS results indicated that metal in the shells is not completely in a carbonate structure, but may also be present in organic material. To the best of our knowledge, such reports on land snail shells have not yet been reported.

A similar existence state was expected for Zn; however, the spectra among parts of the snail bodies did not differ significantly as did the Cu spectra (Fig.1(a), Fig.2(a)). Thus, PLS modelling was not well performed. This is due to the overlapping of sample spectra on O-bound reagents, resulting in poor calculation against the S-bound species. Thus, the comparison of Zn chemical species among each part of snail bodies was not performed. Yet, it was found that the metal is not completely captured by inorganic aragonitic structure of the shells. Zn is possibly partly taken into the organic constituents of the shells.

Thus, we were able to estimate, by applying PLS, the relative abundance of chemical species of metals, especially Cu, in biological samples. This report may be a possible pathway to wider application of XANES and PLS to chemical speciation of environmental samples.

Table 1
Relative abundance of metal species in each part of the snail

		Cu		
		Cu-SOo	Cu-SOi	Cu-S/NO
hepatopancreas	S bound (%)	68	119	99
	O bound(%)	32	-19	1
	residue	0.027	0.032	0.037
body	S bound(%)	78	121	95
	O bound(%)	22	-21	5
	residue	0.040	0.040	0.040
mantle	S bound(%)	57	99	97
	O bound(%)	43	1	3
	residue	0.024	0.030	0.037
shell	S bound(%)	8	46	24
	O bound(%)	92	54	76
	residue	0.055	0.064	0.087

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