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Studies of the uptake and binding of trace metals in fungi. Part I: Accumulation and characterization of mercury and silver in the cultivated mushroom, *Agaricus bisporus*

A R Byrne and M Tušek-Žnidarič
J. Stefan Institute, E. Kardelj University of Ljubljana, 61000 Ljubljana, Yugoslavia

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The champignon mushroom *Agaricus bisporus* was cultivated on compost labelled with the radioactive tracers ^{110m}Ag and ^{203}Hg and the uptake of these two metals studied. The radiotracer then served as an aid in elucidation and characterization of the fungal metal–protein species using gel chromatography. In the case of the silver–protein complex, but not of mercury, the majority of the metal was contained in the fractions of intermediate (*ca* 8000–10000 Da) molecular weight possibly corresponding to metallothioneins (MT) or MT-like species.

Keywords: *Agaricus bisporus*, fungi, mercury, silver, metallothionein, gel chromatography, radiotracters, silver-110m, mercury-203

INTRODUCTION

Although it is now well documented that higher fungi generally have greater trace element contents than green plants, and there are quite a number of species with specific accumulative abilities for particular elements, in general they have attracted less interest as environmental monitors or biological pollution indicators than might be expected. This is especially true in comparison with mosses or epiphytic and epilithic lichens, which have been paid considerable attention as integrating collectors and monitors for both metals and radionuclides. Part of the reason for this neglect may lie in the poorly understood nature of the metal–fungal binding or complex, as well as of the mechanism of mycelium transport. Recently Tomsett and Thurman,[1] in a broader review of metal tolerances in plants, described evidence for the occurrence and properties of metal-binding proteins (metallothioneins) and peptides (phytochelatins) in fungi and plants. In comparison with studies of metal binding in mammals and aquatic species such as molluscs, where the field of metallothionein (MT) or MT-like proteins is being intensively studied, few investigations have been devoted to fungal metal-binding proteins. So far only copper-containing MTs have been isolated and characterized; thus Cu-MT from the mould *Neurospora crassa* has been isolated and structurally elucidated[2,3] and Cu-MT from *Agaricus bisporus* likewise,[4] including the amino-acid sequences, which revealed a considerable degree of homology with mammalian MTs. However, these Cu-MTs were isolated from cultured mycelia, not from natural fruiting bodies.

In the present work, the accumulation of mercury and silver in the fruiting bodies of the cultivated champignon mushroom, *Agaricus bisporus*, was studied using metal-containing compost labelled with ^{203}Hg (half-life 47 days) and ^{110m}Ag (half-life 250 days). The radioactive tracer incorporated in the fungus then served as an aid in elucidation and characterization of the fungal metal–protein complex. Silver was of interest in view of reports of high natural accumulation of Ag in the Lycoperdaceae (puffballs)[5] and in the Agaricaceae[6] (and because of a recent finding of high accumulation of ^{110m}Ag from Chernobyl fall-out in this family[7]).

Gel chromatography was applied in an attempt to characterize the metal–protein species and to look for possible MT or MT-like proteins.

EXPERIMENTAL

1 Cultivation of *Agaricus bisporus*

The mycelium was obtained by courtesy of Droga, Središče ob Dravi, Slovenia, Yugoslavia, and consisted of *Agaricus* sp., Fritsche 217 (Horst America). The compost with mycelium was well mixed in a 50 dm^3 polythene drum with 250 cm^3 of ^{203}Hg-labelled murcury acetate ($HgOAc_2$) solution, or ^{110m}Ag-labelled silver nitrate ($AgNO_3$) solution (see Section 2 below). The samples were kept in a thermostated room at 25 ± 3°C, and the humidity of the compost, which was initially adjusted to 60%, was maintained at 60 ± 3%. After 24 days, when the mycelium had multiplied throughout the compost, a layer of 4–5 cm of sterilized peat of pH 7.5 and 85% humidity was spread over the substrate and kept a further 18 days at 25°C, maintaining the humidity at 75%. The temperature was then lowered to 16–18°C and after about 10 days fruit bodies began to appear.

2 Radioisotopic labelling with ^{203}Hg and ^{110m}Ag

It was known from the work of Aichberger and Horak[8] that *Agaricus bisporus* would tolerate mercury concentrations of at least 10 mg kg^{-1} in the substrate. For silver, which might be more toxic, a somewhat lower substrate concentration was chosen (4 mg kg^{-1} dry weight or 1.5 mg kg^{-1} fresh weight).

Mercuric oxide (HgO) and silver foil were irradiated for 40 h in the rotating rack position of a Triga Mk II reactor at this Institute at a neutron flux of 2×10^{12} n cm^{-2} s^{-1}, and then shorter-lived radionuclides allowed to decay for three weeks. HgO (54 mg) was then dissolved in 1 cm^3 of glacial acetic acid and diluted to 50 cm^3 with water to give a stock mercury solution of 1 mg cm^{-3} labelled with ^{203}Hg (half-life 47 days). Similarly 50 mg of silver foil was dissolved in 1 cm^3 of conc. (15 mol dm^{-3}) HNO_3 and diluted to 50 cm^3, or 1 mg cm^{-3} Ag, labelled with ^{110m}Ag (half-life 250 days).

The stock labelled Hg solution was diluted to 250 cm^3, then added to about 5 kg of compost, and correspondingly 10 cm^3 of stock labelled silver sqlution, diluted to 250 cm^3, was added to a separate batch of compost. The metal contents of the compost were thus 20 mg kg^{-1} (dry) or 7.5 mg kg^{-1} (fresh) for mercury, and 4 mg kg^{-1} (dry) or 1.5 mg kg^{-1} (fresh) for silver.

Two aliquots of compost with mycelium were used without addition of metal-containing radiotracers, for measurement of the blank metal contents of both substrate and *Agaricus bisporus* samples.

The radioactivity of the tracer solutions, the fruit bodies and the protein fractions were measured by gamma-counting in a 3 in × 3 in (7.5 cm × 7.5 cm) sodium iodide (thallium) [NaI(Tl)] detector connected to a 256-channel analyser. The specific activity of both stock solutions at the begining of the experiment was about 1000 cpm μg^{-1} of metal. By reference to an aliquot of the stock solution, the metal concentrations of the samples could be obtained directly from their count rates.

3 Harvesting and treatment of fruit bodies

The fruit bodies appeared first singly and then in flushes or batches at roughly weekly intervals for three or four weeks. These batches were collected separately. The caps and stalks were separated and the caps cut in half, and after weighing, one half was stored frozen at −25°C, and the other half freeze-dried and homogenized. In the case of silver-labelled mushrooms, the stalks were also divided and treated as for the caps. Samples of the freeze-dried mushroom powder were counted under the same geometry as the stock solutions to determine their metal content.

4 Protein separation and analysis

The procedure for preparation of the fungal extract and its protein fractionation by gel chromatography is shown schematically in Fig. 1. The finely chopped fruit body tissue was homogenized in an ice bath for 10 min with two volumes of ice-cold buffer [10 mmol dm^{-3} Tris–HCl, 0.1 mmol dm^{-3} phenylmethylsulphonyl fluoride (PMSF – a protease inhibitor, at pH 7.6] and immediately centrifuged (Sorvall Superspeed RC 2-13) for 45 min at 9700–10000 rpm at 4°C. Gel chromatography was carried out on a 2.5 cm^3 subfraction of the extract on a 60 cm × 1.6 cm Sephadex G-75 column at a flow rate of 13.8 cm^3 h^{-1} using 10 mmol dm^{-3} Tris–HCl at pH 7.6 as eluent. The 5-cm^3 fractions collected were then measured for absorbance at 254 and 280 nm and for radioactivity of ^{203}Hg or ^{110m}Ag. The chromatographic column was previously calibrated under the same conditions with blue dextran (200000 Da), bovine serum albumin (67000 Da), chymotrypsinogen A (25000 Da) and cytochrome *c* (12300 Da).

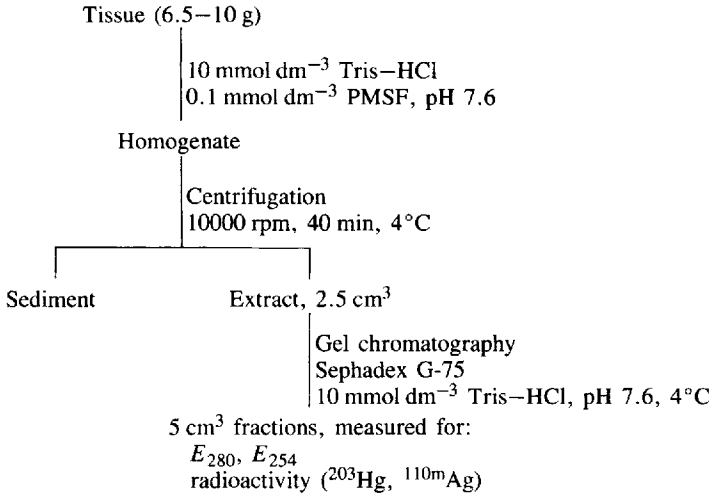

Figure 1 Scheme for protein isolation from fungal tissue.

Table 1 Accumulation of mercury in *Agaricus bisporus*

Sample	Date	No. of specimens	Mass (g, fw)	Hg content[a] (mg kg^{-1}, dry)	CF
1 Cap	29 May 1986	1	50	75.0	3.70
2 Cap	6 June 1986	5	51	30.7	1.53
3 Cap	9 June 1986	7	53	19.2	0.96
4 Cap	17 June 1986	10	106	26.2	1.30

Abbreviations: fw fresh weight; CF, concentration factor (see text). [a]In samples from the blank (unspiked) compost (mercury content 0.06 mg kg^{-1}) the Hg content of *A. bisporus* was 0.09 mg kg^{-1}.

RESULTS AND DISCUSSION

Metal accumulation

Tables 1 and 2 show the details of the accumulation experiments on *Agaricus bisporus* for mercury and silver, respectively. In the case of the substrate containing mercury, the highest concentration factor (CF), defined as the ratio of the metal concentration in the mushroom to that in the substrate, was 3.7 for the first specimen collected; it subsequently fell in later samples. Aichberger and Horak[7] performed accumulation experiments with *Agaricus bisporus* using three different concentrations of mercury in the substrate (0.1, 1 and 10 mg kg^{-1}), and reported CFs of about 3.

In the case of silver-containing compost, we found a much higher uptake (Table 2), with a CF up to 40, and concentrations in cap tissue of over 100 mg kg^{-1} (dry weight). In nature, the Lycoperdaceae were reported[5] to display CFs of up to 300, and for Agaricaceae[6] CFs of *ca* 1000 can be calculated. The ratio of the cap to stalk concentrations for silver was found to be 1.8 ± 0.26, which is in good agreement with the value of 1.7 reported for mercury.[8] This presumably simply represents the relative proportion of binding sites, probably sulphydryl (SH) groups, in the two tissues.

A balance of mercury and silver in the mushrooms and substrate can also be constructed. In the case of mercury the recovery in the mushrooms of metal added to the substrate was about 10%, while for silver no less than 78% was taken up by the fruit bodies. Thus it is not surprising that concentrations of silver declined in the later flushes.

Table 2 Accumulation of silver in *Agaricus bisporus*

Sample	Date	No. of specimens	Mass (g, fw)	Ag content[a] (mg kg^{-1}, dry)	CF[b]
1 Cap	3 June 1986	1	80	167.3	41.5
Stalk			60	79.7	
2 Cap	6 June 1986	1	28	105.0	26.1
Stalk			19	66.8	
3 Cap	11 June 1986	26	360	42.8	10.6
Stalk			240	22.2	
4 Cap	19 June 1986	8	200	27.5	6.8
Stalk			125	13.6	
5 Cap	26 June 1986	1	20	17.5	4.3
Stalk			10	11.7	

[a]In samples from the blank (unspiked) compost the silver content of *A. bisporus* was 0.38 mg kg^{-1}. [b]CF = concentration factor (see text).

Metal–protein investigation

As described above in the Experimental section, the centrifuged extract from homogenized cap tissues was subjected to a standardized gel-chromatographic protein separation procedure. The distribution of radioactivity, and therefore of metal, between the extract and the residue after homogenization and centrifugation was about two-thirds in the extract and one-third in the residue for silver, and slightly more in the residue for mercury.

The results of the experiments on protein separation by gel chromatography are shown graphically in Fig. 2 (mercury) and Fig. 3 (silver), where the fraction number, calibrated as molecular weight in Daltons, is plotted against metal content and absorption at 254 nm (E_{254}) and at 280 nm (E_{280}). (The experiments were repeated for silver-labelled mushrooms using samples from the first and then the second batch.) It can be seen that the curves obtained for silver are quite different from those for the mercury-labelled substrate.

In the case of mercury, the high-molecular-weight fraction contains 60% of the metal, 25% is in a broad shoulder at intermediate molecular weights, with the rest in the low-molecular-weight fractions. In contrast, in the case of silver, the corresponding figures are 20% for the high-molecular-weight fraction and about 65% in the intermediate fraction with a peak at 8000–10000 Da, although the peak is a broad one. This is the range of molecular weight in which metallothioneins (MTs) or their dimers are usually found. MTs are cysteine-rich proteins, capable of binding metals such as copper, zinc and cadmium in thiolate (mercaptide) clusters, consisting of a single polypeptide chain with a molecular weight of about 6000 to 7000. On gel filtration, native metal-containing MT moves like a spherical protein of molecular weight 10000.[9] Mammalian MTs have 61 amino-acid units with 20 cysteines, and lack aromatic groups and histidine. Absorption at 280 nm, E_{280}, is used to indicate the presence of aromatic groups. However, like most biochemical tests, this is not specific; for example Cu-MTs also absorb at this wavelength. The measurement of absorption at 254 nm is also a compromise; what is being sought is the characteristic metal–thiolate (mercaptide) linkage formed by the metal–cysteine complex. However, the thiolate complexes of individual metals with MT each have their own characteristic absorption wavelengths; at around 220 nm for zinc, 250 nm for cadmium and 270–280 nm for copper,[9] although the peaks are not sharp or well-defined.

It should be appreciated that the presence of MTs cannot be unequivocally established from such gel-chromatographic separations as the present one, since the cysteine groups are not directly detected but only inferred from the presence of metal-containing peaks at the appropriate molecular weight, and from the shape of the E_{254} and E_{280} curves. Further experiments involving additional purification, separation and amino-acid sequencing are required. In the case of the silver-containing fraction from *Agaricus bisporus* with a peak at 8000–10000 Da, this peak and the general shape of the chromatograph is certainly interesting enough to warrant further investigations and characterization of this type.

Further parallel experiments involving cadmium would be instructive (^{109}Cd could be used as a

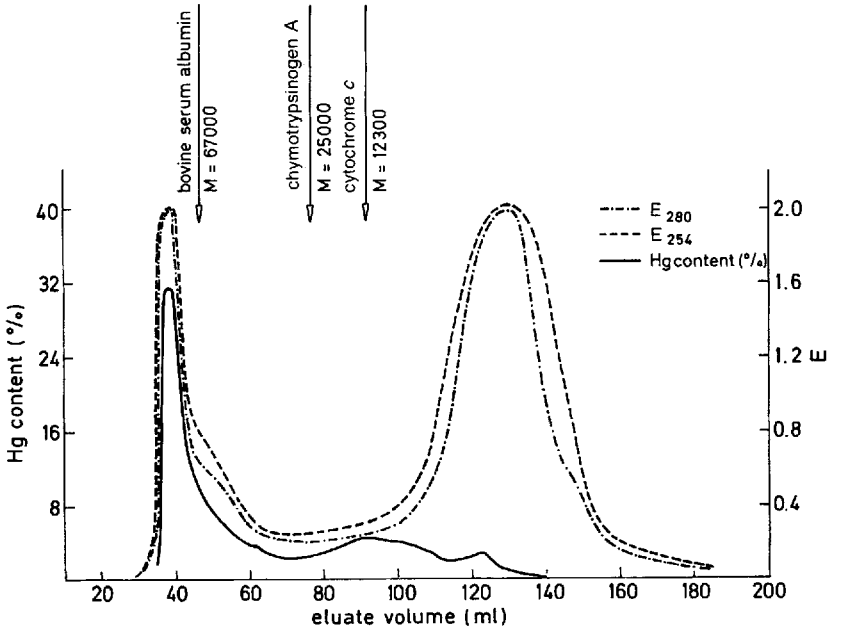

Figure 2 G-75 Sephadex gel chromatography of extract from mercury-labelled *Agaricus bisporus*.

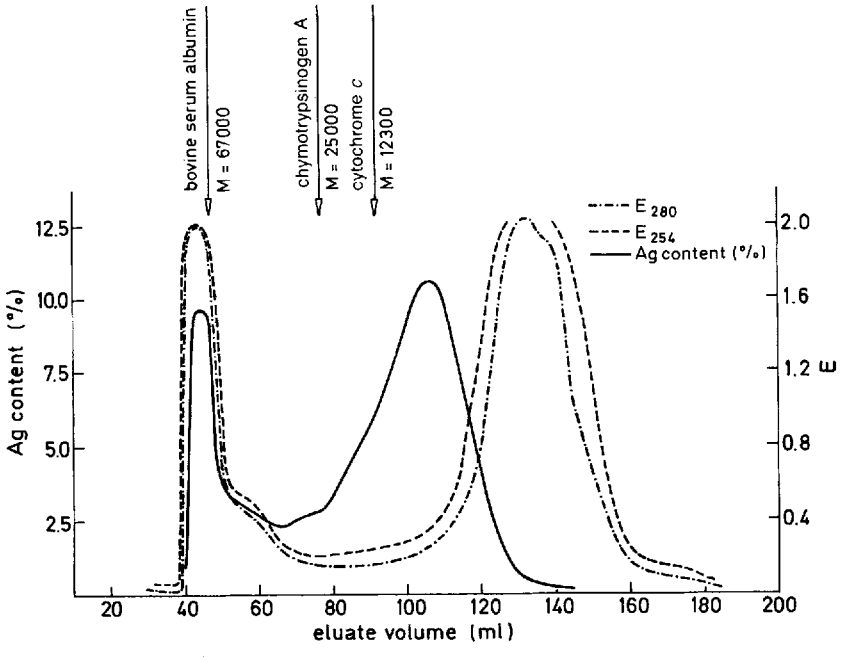

Figure 3 G-75 Sephadex gel chromatography of extract from silver-labelled *Agaricus bisporus*.

radiotracer with suitable properties). Earlier work in our laboratory on the characterization of the nature of the protein binding of cadmium in the fly agaric, *Amanita muscaria* (which contains naturally 10–15 mg kg^{-1} of cadmium[10]) by Stegnar[11] showed that most of the cadmium was associated with proteins in the 30000–50000 molecular weight range. Cadmium uptake by several edible mushrooms of the Agaricaceae and Boletaceae is high[10] and had led to public health recommendations in the FRG to limit consumption of wild mushrooms.

Thus studies on cadmium binding in *Agaricus bisporus* would be of importance in their own right, as well as helping in the interpretation of the present and future more detailed studies on silver.

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