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Characterization Studies on Cadmium–Mycophosphatin from the Mushroom *Agaricus macrosporus*

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A low molecular weight Cd-binding phosphoglycoprotein, cadmium-mycophosphatin, has been isolated from the mushroom *Agaricus macrosporus*. This protein has a molecular weight of 12,000 dalton and contains no sulfur but a high amount of acid amino acids (Glu, Asp), and carbohydrates (glucose, galactose). Cadmium-mycophosphatin has an isoelectric point less than pH 2, binds cadmium with a dissociation constant of K_D = 1.59 × 10 M (pK_D = 6.8) and is saturated with 13.5 mole Cd/mole, all Cd-binding sites being equivalent. It is suggested that Cd is bound by phosphoserine groups, similar relations being known from calcium-binding proteins in animals. From *A. macrosporus* four other low-molecular weight glycoproteins have been isolated which contain sulfur and bind cadmium and copper. The biological significance of these Cd-binding proteins is discussed.

Introduction

In 1976 the German news magazine, *Der Spiegel*, reported that mushrooms from the market, especially those of the genus Agaricus ("champignons"), sometimes contained extraordinary high amounts of the toxic metal cadmium (*1*). This information came from an official laboratory of the city of Saarbrücken, where foods are analyzed as a matter of routine. Since the taxonomic relations of these mushrooms under investigation had not been clearly defined, we analyzed 28 species of Agaricus and 52 other European mushroom species from several genera on their content of cadmium, zinc, and copper and found that some species especially from the genus Agaricus are able to accumulate very specifically the metal cadmium (*2*). This phenomenon proved to be of taxonomical value and was not the result of environmental contamination with cadmium from the soil (*2*). Cd concentrations of these mushrooms were found in some cases to exceed 100 mg/kg dry weight compared to about 1 mg Cd/kg or less in other mushrooms (*2,3*).

Since Cd-rich mushrooms are able to accumulate the metal up to 300-fold compared to the soil (*2*), cadmium should be of special biochemical importance for these organisms. This was studied with isolated mycelium of *Agaricus abruptibulbus*, because fruit bodies of this species of the Agaricaceae do not grow under definite conditions in the laboratory.

It was found Cd-free grown mycelium of *A. abruptibulbus* increases its growth up to 100% in the presence of 0.5 mg Cd/L nutrient medium (*4*). This growth stimulation was not correlated with zinc supplementation, and Cd absorption by the cells was found to be independent from zinc in such a way that a special Cd transport system was postulated for this mushroom (*4*).

Further work now concentrates on Cd-binding within the mushrooms in order to learn more about the biochemical function of cadmium in these organisms.

Materials and Methods

Frozen fruit bodies of *Agaricus macrosporus* which had been collected in the Saarland, F.R.G., were homogenized mechanically in 20% aqueous trichloroacetic acid (TCA) and extracted for 24 hr at 0°C. After centrifugation (27,000*g*, 30 min), the supernatant was extracted three times with about one-third of its volume of diethyl ether in order to remove the TCA. The aqueous phase was then lyophilized and further used for the separation procedure.

The isolation of Cd-binding proteins was performed by several gel filtration steps on Sephadex G-25 and DEAE–Sepharose CL-6B as described earlier (*5*). Metal analyses were performed by atomic absorption spectroscopy (Perkin-Elmer 420 with graphite furnace HGA-74).

Amino acids in protein hydrolyzates were determined in an amino acid analyzer 4400 from LKB, Bromma, Sweden. Carbohydrates were detected and analyzed in protein hydrolyzates as described elsewhere (*5*).

Detailed descriptions for the estimation of Cd-binding constants and for the electrophoretic procedures are given by Meisch et al. (*5*).

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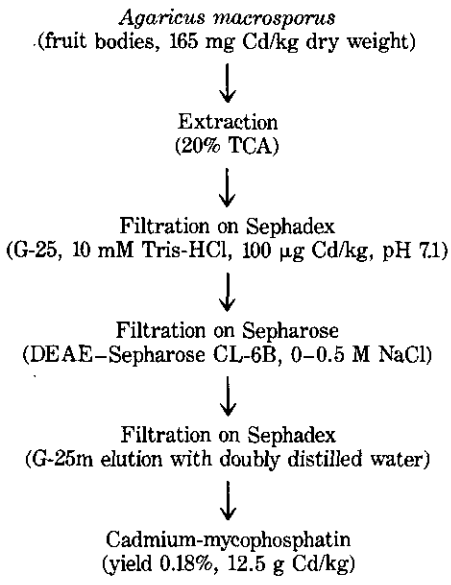

FIGURE 1. Scheme for isolation of cadmium-mycophosphatin from the mushroom *Agaricus macrosporus*.

Results and Discussion

The big-spored whitecap *Agaricus macrosporus*, a mushroom species which is known to accumulate an appreciable amount of cadmium from the soil (*2*), was collected on meadows in the Saarland region of Germany and used for the isolation of cadmium-binding compounds. An average content of 165 mg Cd/kg dry weight was determined in the fruit bodies. As known from preliminary experiments (*6*), extraction with 20% aqueous TCA is suitable for an efficient separation of the Cd-binding components from bulk mushroom material and from interfering proteins. After removal of the TCA by extraction with diethyl ether, the aqueous phase contained 46% of mushroom dry weight and 87% of the fungal cadmium. Filtration of the lyophilized extract on a Sephadex G-25 column resulted in a protein fraction which contained more than 60% of the eluted cadmium. This fraction was further purified by chromatography on DEAE–Sepharose CL-6B and on Sephadex G-25 according to the scheme shown in Figure 1. In order to prevent a substantial loss of cadmium from the protein, the first filtration steps were always run in the presence of Cd-acetate (100 μg Cd/kg of buffer solution).

Preliminary tests showed that the purified Cd-containing compound is a low molecular weight protein which contains also carbohydrates and phosphorus but no sulfur. We therefore proposed the name cadmium-mycophosphatin (*5*).

Cadmium–mycophosphatin was now characterized by a variety of methods.

Characterization

Thin-Layer Electrophoresis. On borate-buffered cellulose or silica gel, Cd–mycophosphatin migrates to the anode and can there be stained with Alcian Blue or with Toluidine Blue after fixation with acetic acid/ethanol/water (10:10:80, v/v).

Table 1. Cadmium-mycophosphatin: general properties.

Property	Value
Molecular weight	12,000 daltons
UV spectrum	
λ_{max}	260 nm
ε	24,600
Isoelectric point	< pH 2
Elementary composition	
C	37.19%
H	5.23%
N	5.14%
P	3.71%
S	0.0%
Residue	12.8%
Amino acids	15, no S-containing acids
Carbohydrates	
Glucose	9.9%
Galactose	7.3%
Cadmium content (at saturation)	13.5 mole Cd/mole protein
Stability of the Cd bond	$K_D = 1.59 \times 10^{-7}$ M; $pk_D = 6.8$

Ultrathin-Layer Isoelectric Focusing. The Cd-protein migrated together with the front markers Bromphenol Blue or Fast Green CF to the anode; it was not focused in the applied range of pH 2.4 to 6.6.

Two-Dimensional Electrophoresis. Cd-mycophosphatin always stayed on the anodic side of the application zone in the range of pH 2 to 9. No isoelectric point could therefore be detected down to pH 2.

Gel Filtration. The apparent molecular weight of Cd-mycophosphatin was determined on a Sephadex G-50 column which had been standardized by running the test proteins RNAase, cytochrome c, aprotinin, insulin B-chain, and bacitracin. Compared to a calibration curve which had been set up with these proteins, the molecular weight of Cd-mycophosphatin was calculated to 12,000 daltons.

Ultraviolet Spectrum. Cd-mycophosphatin showed a very simple ultraviolet spectrum with a weekly impressed maximum at 260 nm (ϵ = 24,600 at pH 7.1). Only small alterations were observed in the range pH 1 to 13.

Amino Acid Analysis. Hydrolysis of Cd-mycophosphatin and quantitative amino acid analysis indicated the presence of 15 amino acids (Glu, Asp, Gly, Ser, Ser-P, Ala, Pro, Lys, Thre, Val, Leu, Arg, His, Ile, Phe), of which the predominant ones were glutamic acid (20.3 mole-%), glycine (16.5 mole-%), and aspartic acid (15.0 mole-%).

Carbohydrates. Investigation of the hydrolyzate indicated the presence of glucose and galactose. The glucose content (9.9 mole-%) was determined enzymatically (hexokinase/glucose-6-phosphate dehydrogenase), while galactose (7.4 mole-%) was analyzed by gas chromatography of the silylated sugar.

A summary of the characteristic properties of Cd-mycophosphate is given in Table 1.

Other Methods. The fungal protein was further investigated by ^{1}H-, ^{13}C-, and ^{31}P-NMR spectroscopy. In

Table 2. Other metal-binding proteins from *Agaricus macrosporus*.

Protein	Molecular weight	Metal content, mg/kg		S, %	P, %	Main amino acids
		Cu	Cd			
AM-1,1	3300	1232	424	0.68	0	Glu, Gly, Asp, Ser
AM-1,2	4900	3017	901	1.42	1.95	Glu, Gly, Asp, Ser
AM-2	5900	2040	444	3.82	2.92	Glu, Gly, Asp, Ser
AM-3	4000	1300	819	0.39	19.6	Glu, Gly, Asp, Ser

[a]Carbohydrates: 10–20% as *N*-acetylglucosamine.

agreement with the biochemical data, the ^{1}H- and ^{13}C-spectra reveal a sugar component and a protein moiety with low aromatic side chain content. The sugar resonances clearly dominate these spectra with relative narrow lines and high intensity, while the protein sidechain resonances show broader lines and low intensity. The ^{31}P-NMR spectrum is quite interesting: relative to H_3PO_4 as a standard, the phosphorus atoms of the phosphoserine groups occur over a range of chemical shifts, indicative of a number of nonequivalent environments for the phosphorus side chains in the protein structure. According to the NMR spectra, CD-mycophosphatin may have a tightly folded, relative immobile protein core (broad lines, low intensities) and a highly mobile, flexible sugar coat (narrow lines, full intensities). The phosphate groups would be nonequivalent in the folded protein structure and still exhibit narrow lines due to internal mobility in the side chain (A. Ribeiro, personal communication).

Stability of the Cadmium Binding

The binding behavior of cadmium in the fungal protein was investigated by equilibrium dialysis against several buffers in the range pH 4.0 to 10.4 Independent of the buffer system used, cadmium is most tightly bound beyond pH 8, while at pH 7, less than 50% of the metal is still attached to the protein. The stability of the Cd-binding and its stoichiometry were determined by equilibrium dialysis using the buffer system Tris-HCl (5 mM) at pH 8.5. From Scatchard plots, a dissociation constant $K_D = 1.59 \times 10^{-7}$ M ($pK_D = 6.8$) was calculated, while Cd-mycophosphatin was found to be saturated with cadmium by $n = 13.5$ mole Cd/mole of protein (*5*).

According to the overall properties mentioned above, Cd-mycophosphatin represents a new type of naturally occurring Cd-binding proteins which differs substantially from the widely distributed metallothioneins in its molecular structure and in its Cd-binding ability. The main difference is the lack of sulfur in the fungal protein, while about 40% of the molecule is built up by acid amino acids (Glu, Asp) and of phosphoserine. Since SH-groups are absent, the phosphates of phosphoserine might be responsible for cadmium-binding. This is supported by the low pK_D of 6.8 (metallothioneins bind cadmium and have pK_D values of about 17) (*7*) and by the comparison of the total serine + phosphoserine content (14 mole/mole of protein) with the phosphorus content (14 mole/mole) and the molar Cd-concentration at saturation (13.5 mole Cd/mole). In addition, the titration characteristics show an inflection point near pH 7.5 (*5*) which points to a participation of monophosphate esters in Cd-binding. Similar relations are known from Ca-binding phosphoproteins from rat teeth (*8*) or egg yolk (*9*), where the dissociation constants of Ca^{2+} have the same order of magnitude and where no isoelectric point can be found beyond pH 2.

A comparison of the Cd content of *Agaricus macrosporus* (165 mg/kg dry weight) with the yield of isolated Cd-mycophosphatin (0.2%) shows that the protein is not saturated with cadmium. In fact, there are some other proteins in the mushroom which compete with Cd-mycophosphatin for Cd-binding. It was shown that up to 32% of the native cadmium may be bound in *A. macrosporus* at a protein fraction of high molecular weight (*10*). On the other hand, four other low molecular weight glycoproteins have been recently isolated from the same mushroom which bind cadmium, but also copper (*11*). Some of their molecular properties are listed in Table 2.

Table 2 shows that these proteins are acid, too, but they all contain sulfur and therefore seem to have a higher affinity to copper than to cadmium.

In spite of our present knowledge about the molecular properties of Cd-binding proteins from Agaricus, nothing is known about their biological functions in these mushrooms. From recent studies we know that a Cd-mycophosphatin-like substance occurs in the related species *A. abruptibulbus*, a mushroom which also accumulates cadmium, the protein being located in both mycelium and fruit bodies (*12*). From *A. bisporus*, however, which does not accumulate the metal, the absence of low molecular weight metal-binding protein is reported (*13*). So we assume that the phenomenon of Cd accumulation in a subdivision of the genus Agaricus may be related to the occurrence of Cd-mycophosphatin or similar proteins with close relationships to the fungal metabolism, e.g., Cd uptake or Cd transport, or even to a function of cadmium as a growth factor for these mushrooms (*4*).

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