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The association mode of arsenic accumulated in the freshwater alga *Chlorella vulgaris*

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Arsenic accumulated in living *Chlorella vulgaris* cells was solvent-fractionated with chloroform/methanol (2:1), and the fractions were analyzed for arsenic. A large part of the accumulated arsenic was localized in the extract residues.

The extract residue from the same extraction of *C. vulgaris*, which had been, however, cultured in any arsenic-free Detmer medium (MD), adsorbed arsenic physico-chemically at a concentration of 1.1 mg As g^{-1} dry weight.

Arsenic was found to be combined with protein with molecular weight around 3000 in the arsenic-accumulated living cells. The arsenic-bound protein was analyzed for amino acids. The experimental results showed that no metallothionein-like protein was inductively biosynthesized in *C. vulgaris* on the exposure to arsenic.

Keywords: Arsenic, methylarsenic accumulation, freshwater alga, *Chlorella vulgaris*, association mode, metallothionein

INTRODUCTION

In a previous paper,[1] five freshwater algae which had accumulated arsenic were solvent-extracted with chloroform/methanol (2:1) and the fractions were analyzed for arsenic. Calculation of data from the previous experimental results showed that the percentages of total arsenic in the algae which was localized in the extract residues from the arsenic-accumulating algae *Chlorella* sp., *Hydrocolium* sp., *Phormidium* sp., *Nostoc* sp., and *Microchaete* sp. were 93%, 91%, 99%, 98% and 86%, respectively.[1] No findings on the association mode of arsenic in these algae were obtained from the above experiments.

A metallothionein-like protein was found to be inductively biosynthesized in *C. vulgaris* which had been exposed to cadmium.[2,3] No information has been reported on the inductive biosynthesis of metallothionein-like proteins in the case of arsenic exposure.

In this report, we discuss experimental results on the distribution of arsenic bioaccumulated in the *Chlorella* cell tissues, the adsorption of arsenic by the cell tissues *in vitro*, gel-filtration chromatography of arsenic-bound proteinaceous material, and amino-acid analysis of the arsenic-bound proteins.

EXPERIMENTAL

General procedure of algal culture

C. vulgaris was cultured and harvested under the conditions described in the previous paper.[4]

Solvent fractionation with chloroform/methanol (2:1) of *C. vulgaris* accumulating arsenic

C. vulgaris was inoculated in 20 dm^3 of modified Detmer medium (abbreviated as MD medium)[4] containing 100 μg g^{-1} of arsenic (as elemental arsenic from Na_2HAsO_4, abbreviated as AS(V)), cultured for 14 days under illumination (6000 lux, 24 h day^{-1}) and harvested by the general procedure.

The wet living cells (2.12 g, on a dry weight basis) were homogenized with chloroform/methanol (2:1) using a Tefron homogenizer (Potter–Elvejhem type), the slurry was filtered under reduced pressure through a filter paper (No. 5C, Toyo Filter Paper Co. Ltd), and the residue was washed with the mixed solvent until the filtrate became colorless. The filtrate was combined with the washings and shaken with one-quarter of their total volume of water, the mixture was allowed to stand at room temperature overnight, and the upper phase (water-soluble) and the lower (lipid-soluble) were separated and evaporated to dryness. The whole cells, the residue, water-soluble and lipid-soluble fractions

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were analyzed for inorganic arsenic and methylarsenic by previously reported methods (viz. hydride generation, GC AA).

Solubilization of proteins in the residue of the solvent extraction

In order to investigate the association mode of arsenic with proteins in the residue of a chloroform/methanol (2:1) extraction, the barely soluble proteins in the residue were solubilized by a standard method using a surface-active agent described below.[5]

The residue (*ca* 100 mg) was pulverized, mixed with 1% sodium dodecyl sulfate (SDS) (membrane-protein solubilizer: SPS-4, Nacali Tesque Co. Ltd) (15 cm^3, pH 8.6) and allowed to stand at 40 °C for 24 h. The suspension was centrifuged, the supernatant was concentrated by a rotary evaporator at a reduced pressure and a condensed protein solution was obtained.

Determination of proteins

Protein in the above solubilized-protein solution was determined by the method of Lowry *et al.*[6] as follows.

The protein solution (0.1 cm^3 containing 5–100 μg protein) was mixed with 1 cm^3 of 2% Na_2CO_3 (in 0.1 mol dm^{-3} NaOH)/0.5% $CuSO_4$ (in 1% sodium tartrate) (50:1), the mixture was allowed to stand for 10 min and mixed with Folin–Ciocalteu reagent solution[7] ('phenol reagent solution', acidity 1.8N; Nacali Tesque Co. Ltd, Japan) with rapid agitation. After the mixture had been left to stand for a further 30 min, protein was determined by spectrophotometry at 750 nm.

Gel-filtration chromatography of solubilized proteins

Solubilized arsenic-bound proteins were fractionated to their molecular weights by gel-filtration chromatography by use of Sephadex G-75 (Pharmacia LKB Biotechnology; 40–120 μm diameter; fractionable molecular weight (MW) ranging from 3000 to 80 000). Sephadex G-75 column (2.0 cm i.d., 80 cm long) was preconditioned with an eluent solution of 0.1% SDS in 10 mM-Bicine (Good buffer; *N*,*N*-bis(2-hydroxyethyl)glycine; Dojindo Laboratories, Japan) (pH 8.6). The clear aqueous protein solution was put on the column and eluted with the eluent at a flow rate of 1 cm^3 min^{-1}. The eluates were collected by a fraction collector (200 drops, ca 4.5 cm^3 each) and the fractions were analyzed for arsenic and protein. Total arsenic was determined by flameless atomic absorption spectrophotometry. Protein was determined both by the above-mentioned Lowry method and by a UV method at 254 nm. The molecular weight of the protein was calibrated with standard polystyrene sulfonate samples (MW 6500, 16 000 and 31 000). The plots of the retention volumes versus the molecular weight of the standard polystyrene sulfonate samples showed good linearity.

Amino-acid analysis of proteins

Dry powdered protein was mixed with 10 cm^3 chloroform, the mixture was filtered on a 4.5 μm membrane filter and the filtrate was concentrated in vacuum to give a white powder. The powder was dissolved with 6 mol dm^{-3} HCl and the protein solution was hydrolyzed by heating at 110 °C in a sealed tube for 22 h. The hydrolyzed amino-acid solution was heated to dryness, the dry powder was dissolved with 0.5 cm^3 citric acid buffer (pH 2.2), and the insoluble matter was removed by filtration on a 0.5 μm membrane filter. The filtrate was analyzed for amino-acids using an automatic amino-acid analyzer (JASCO 801-SC; detector, Hitachi 650-10S).

RESULTS AND DISCUSSION

Solvent fractionation of *C. vulgaris* accumulating arsenic

C. vulgaris was cultured for 14 days in MD medium containing 100 μg As(V) g^{-1} by the general procedure and the arsenic-accumulated algal cells were harvested. The wet algal cells were fractionated with chloroform/methanol (2:1) by the method described above.

The experimental data on yields and arsenic concentrations of fractions obtained from the fractionation of *C. vulgaris* accumulating arsenic are summarized in Table 1.

Table 1 shows that a large part (96%) of arsenic accumulated by the alga was localized in the extract residue and that almost all of the arsenic in the residue existed in the inorganic form. The relative gross weights (%) of arsenic (percentages for the original cell) in the water-soluble and

Table 1 Solvent[a] fractionation of living cells of *C. vulgaris* accumulating arsenic

		Arsenic ($\mu g\ g^{-1}$)				
Fraction	Yield (g)	Total	Inorganic	Monomethyl	Dimethyl	Trimethyl
Original cell	2.12 (100)[d]	860 (100)[c]	850 (98.8)[c]	2.7 (0.3)[c]	7.3 (0.8)[c]	Trace
Residue	1.91 (89.9)[d]	919(100)[b] (96)[c]	916(99.7)[b]	Trace	2.8(0.3)[b]	Trace
Water-soluble	0.02 (0.8)[d]	833(100)[b] (0.7)[c]	675(77.5)[b]	67(8.0)[b]	121(14.5)[b]	Trace
Lipid-soluble	0.18 (9.3)[d]	307(100)[b] (3.0)[c]	276(90.0)[b]	Trace	31.2(10.0)[b]	Trace

[a] Chloroform/methanol (2:1). [b] %, for fraction. [c] %, for original cell. [d] %.

lipid-soluble fractions were 0.7% and 3%, respectively, being very small.

However, the relative concentrations of methylated arsenic compounds in the water-soluble (22.5%) and lipid-soluble (10%) fractions were found to be much larger than that in the residue (1.1%).

It is known that living *Chlorella* cells consist of 55% protein, 18% lipid, 18% carbohydrate, 6% ash and 3% H_2O on a dry weight basis, calculated from numerous literature sources.[9] The residue of the above chloroform/methanol (2:1) extract probably includes most of the protein (55%), carbohydrate (18%) and ash (6%), and a part of the lipid fraction (18%); 96% of the arsenic distributed in the residue could be combined with any of these components.

Adsorption of arsenic by the residue of the chloroform/methanol (2:1) extract of arsenic-free cells of *C. vulgaris*

Wet cells of *C. vulgaris* (914 mg on a dry weight basis) cultured in an arsenic-free MD medium were extracted with chloroform/methanol (2:1) in the same way as described above; a part of the extract residue (400 mg) was placed in a glass column (7.5 mm i.d., 37 mm column height). A mixed solution of chloroform/methanol/water (3:48:47) containing Na_2HAsO_4 [abbreviated as As(V)] at the level of 1.8 μg As(V) g^{-1} was eluted at a flow rate of 0.3 $cm^3\ min^{-1}$ until the arsenic concentration of the eluate reached the original [1.8 μg As(V) g^{-1}].

Figure 1 shows the breakthrough curve in the column operation. The adsorption capacity (μg of adsorbed arsenic per g dry residue) was calculated from the curve to be 1100 $\mu g\ g^{-1}$. The value of the arsenic adsorption capacity of the residue *in vitro* is approximated by the value of arsenic bioaccumulation of the residue *in vivo* (919 $\mu g\ g^{-1}$) as shown in Table 1.

When the same mixed solvent but containing 1 mol dm^{-3} hydrochloric acid in the place of arsenic (i.e. no arsenic) was eluted through the residue column which had adsorbed arsenic previously, 24% of the adsorbed arsenic was desorbed. The remainder (76%) was found to be so tightly combined with the residue components that the remainder could not be dissociated by 1M hydrochloric acid.

These experimental data showed that although previous reports show no arsenic is accumulated by either heat-killed or dinitrophenol (respiratory

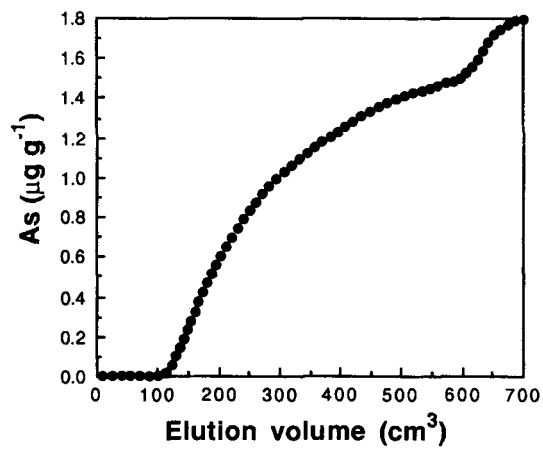

Figure 1 Adsorption of arsenic(V) *in vitro* on the residue, after extraction with $CHCl_3$/MeOH (2:1), of arsenic-free *C. vulgaris* cells. Column: arsenic-free extraction residue (400 mg) filled in glass tube (7.5 cm × 37 mm). Eluent: $CHCl_3$/MeOH/H_2O (3:48:47) containing 1.8 $\mu g\ g^{-1}$ arsenic(V).

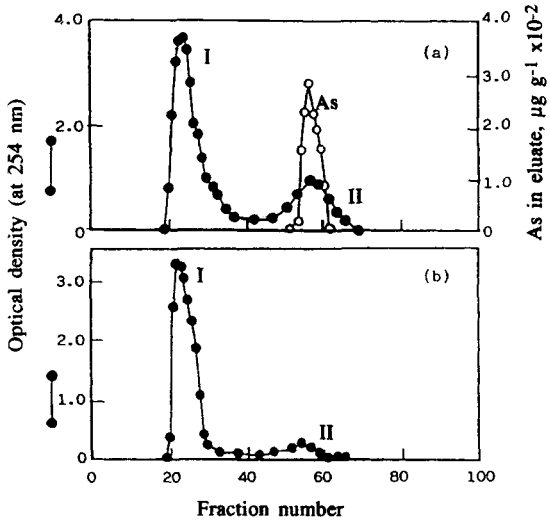

Figure 2 Gel-filtration chromatography on Sephadex G-75 of proteins separated from arsenic-accumulated (a) and arsenic-free (b) *C. vulgaris* cells. Peaks I and II correspond to proteins having molecular weights of approximately 2×10^4 and 3×10^3, respectively.

inhibitor)-treated *Chlorella* cells,[8] the cell components of the alga, such as proteins or carbohydrates, adsorbed arsenic in this case *in vitro* at a level comparable with that of bioaccumulation.

Analysis of arsenic-bound proteins in arsenic-accumulated *C. vulgaris*

Living *C. vulgaris* which had been cultured in the MD medium containing 1000 mg dm^{-3} of arsenic(V) and which had accumulated arsenic at a level of 8700 μg As g^{-1} (on a dry weight basis) was fractionated with chloroform/methanol (2:1) in the same manner as described before. An extract residue containing 7400 μg As g^{-1} (on a dry weight basis) was obtained.

Proteins in the extract residue was separated by the solubilization technique, determined and fractionated on the basis of molecular weight by the methods described in the Experimental section.

In the same manner, arsenic-free proteins were separated from the extract residue of arsenic-free *C. vulgaris* cells.

Gel-filtration chromatograms of the solubilized proteins from arsenic-bound and arsenic-free residues are shown in Fig. 2.

The peak positions of proteins in Fig. 2 obtained from the determination by the UV method completely coincided with those by the Lowry method.[6]

The chromatogram (Fig. 2a) has two peaks (I and II) of protein and one peak of arsenic, and the chromatogram (Fig. 2b) has two peaks of protein only. The fraction numbers of the two

Table 2 Amino-acid composition of arsenic-bound protein and arsenic-free protein in this study, and data from literature

	Amino-acid composition (mol %)			
Amino-acid	As-bound protein[a]	As-free protein[a]	Cd-bound protein[3]	*Chlorella* in literature[9]
Glycine	22.1	11.1	0.84	10.6
Alanine	14.1	19.8	5.42	8.76
Valine	6.64	7.12	2.40	6.42
Leucine	1.53	2.97	4.88	8.67
Isoleucine	1.36	2.00	4.06	5.61
Serine	11.4	5.04	6.79	5.13
Threonine	3.92	6.01	2.74	5.22
Cysteine	1.02	0.76	8.31	0.96
Methionine	0	2.70	0.43	1.30
Aspartic acid	0	6.08	0.03	9.14
Glutamic acid	14.1	8.78	7.34	9.36
Arginine	3.41	5.74	5.71	5.82
Lysine	3.07	3.59	1.34	6.38
Histidine	2.90	1.52	1.13	1.54
Phenylalanine	1.87	5.60	2.93	4.42
Tyrosine	0.51	2.35	3.37	2.50
Tryptophan	0	0	1.54	1.08
Proline	12.1	8.71	42.1	7.05

[a] In this study.

protein peaks in the chromatogram (a) coincide with those in the chromatogram (b), respectively. The height of peak (II) in the former was larger than that in the latter.

It was found from the calibration curve obtained by the use of molecular weight (MW)–standard polystyrene sulfonate that the two protein peaks in these chromatograms correspond to MWs of about 2×10^4 and 3×10^3, respectively. The peak position of protein (II) with MW 3×10^3 coincides with that of arsenic in chromatogram (a).

These experimental results reveal that the solubilized protein had two types of proteins with MWs around 2×10^4 and 3×10^3 and that the accumulated arsenic was associated with the smaller-MW protein, and also suggest that when arsenic was accumulated in *C. vulgaris* cells, the arsenic associated with the smaller protein increased relatively in the cell.

Amino-acid analysis of proteins

The eluates of peaks (II) in Fig. 2(a) and (b) were collected and analyzed for amino-acids by the method described earlier.

Table 2 shows experimental results of amino-acid analyses of the two protein fractions obtained in this study together with reference data. In Table 2, the data in the third column were quoted from our previous paper,[3] obtained from a cadmium-bound protein which was fractionated in the same way from *C. vulgaris* accumulating cadmium. The literature data in Table 2 were averaged from 20 papers cited.[9]

In comparison with the arsenic-free protein and the literature in Table 2, the contents of glycine, serine, histidine and prorine were higher, and those of leucine, isoleucine, phenylalanine and tyrosine were lower, in the arsenic-bound protein. No methionine, aspartic acid or tryptophan was detected. The meaning of this difference in the content of amino-acids between arsenic-bound and arsenic-free proteins is interesting but it is obscure in this stage.

The most interesting subject now is whether a metallothionein-like protein is inductively biosynthesized in *C. vulgaris* or not on exposure to arsenic. In the case of exposure to cadmium,[3] cysteine-rich protein was biosynthesized by *C. vulgris* (this is shown in the third column in Table 2). However, the cysteine content (1.02 mol %) in the arsenic-bound protein was close to that in the arsenic-free protein in this study and also close to the average from the literature. This experimental result leads to the conclusion that no arsenothionein-like protein was biosynthesized by *C. vulgaris* on exposure to arsenic. It may not be necessary for *C. vulgaris* to biosynthesize metallothionein-like proteins, because the alga may have another detoxifying process for arsenic such as the methylation of arsenic.

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