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Mechanism of Cadmium Accumulation into the Cell of Cadmium-Ion Tolerant Yeast

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카드뮴 내성 효모의 세포내 카드뮴 축적 기작

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The mechanism of intracellular accumulation of cadmium in a cadmium-ion tolerant yeast, *Hansenula anomala* B-7, which is an extreme cadmium tolerant strain and has the ability to take up a large amount of cadmium was investigated. The amounts of cadmium taken up by the scalded yeast cells were 2 to 3 times more than the value of the living cells. The living *Hansenula anomala* B-7 cells adsorbed 74% of cadmium taken up onto the other layer of the cells and 26% of it accumulated inside the cells. But the scalded cells adsorbed 98.3% of cadmium taken up and accumulated 1.7% of it inside the cells. A cadmium uptake and its accumulation were accelerated up to 162.3% and 275.4% by Triton X-100 in the living cells, respectively. Whereas in the scalded cell cadmium uptake was not affected by Triton X-100. Furthermore the cadmium uptake and its accumulation were strongly inhibited by metabolic inhibitors like 2,4-dinitrophenol, sodium azide and potassium cyanide in the living cells, but in the scalded cells cadmium uptake was not affected by metabolic inhibitors. These results suggested that the intracellular accumulation of cadmium by the cadmium-tolerant *Hansenula anomala* B-7 cells was apparently dependent of biological activity, and also gave evidence of the existance of energy-dependent system.

Cadmium is very toxic to eukaryotic microorganisms. Especially cadmium about 100 times more toxic than cobalt to *Saccharomyces cerevisiae* (1), and it inhibited RNA synthesis at 100 μM in mouse liver nuclei (2). Metal ions uptake by microorganisms generally occurs in two phases; one is adsorption caused by a rapid binding on cations to negatively-charged groups on the cell surface, and the other is intracellular accumulation caused by a metabolism-dependent mechanism. The starved cells of *Candida utilis* (3) accumulated zinc by two different processes. The first was a rapid, energy-and temperature-independent system that represented binding to the cell surface and the secondary process was accumulation into the cells by an energy-dependent system. Cadmium uptake by *Escherichia coli* K-12 (4) occurred by means of an active transport system. Furthermore the cadmium accumulation was both energy dependent and temperature sensitive, but zinc uptake did not occur by an active transport system. *Saccharomyces cerevisiae* (5) accumulated cobalt and cadmium by two processes; the first was metabolism independent binding to the cell surface and that was followed by a metabolism-dependent process into the cell. Joho (6) reported that a cadmium-sensitive *Saccharomyces cerevisiae* took up almost all of the cadmium bound to insoluble materials, while the cadmium-resistant yeast took up cadmium in the cytosol and bound it to pro-

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tein of low molecular weight (<30,000). The problems of distribution of the heavy metal ion, cadmium, are important for the elucidation of the biological function of the toxic element and for detoxification mechanisms. In the previous papers, the authors isolated and characterized *Hansenula anomala* B-7, an extreme cadmium tolerant yeast from the sludge of a zinc mining district (7, 8). This yeast took up 34.17 mg of cadmium per gram of dry cells (7) and cadmium accumulation was stimulated up to approximately 40% when the yeast was grown in aqueous medium containing 100 μg/m*l* of cadmium and 0.1% of Triton X-100 at 30°C for 24 hours with shaking cultivation (9). In this paper, the authors examined the adsorption *vs.* accumulation ratios of cadmium taken up by the living or the scalded *Hansenula anomala* B-7 cells and also deduced the mechanism of intracellular accumulation of cadmium into the cadmium tolerant yeast cells used in this study.

Materials and Methods

Organism

Hansenula anomala B-7 that was isolated and identified as an extreme cadmium tolerant yeast by the present authors (7, 8) was used throughout this work.

Preparation of living and scalded *Hansenula anomala* B-7 cells

The culture method of the organism and the preparation of the living cells (as intact cells) were described in the previous paper (10).

Hansenula anomala B-7 cells suspended in saline solution were heated for 5 min in boiling water bath and cooled in cold water in order to prepare scalded cells.

Our preliminary experiment showed that the amounts of cadmium taken up by the scalded cells were almost the same in the range of heating time from 3 to 30 min.

Uptake of cadmium by the living cells

The living or the scalded yeast cells were suspended in 100 m*l* of saline solution containing 100 μg/m*l* of cadmium ion and 0.1% of Triton X-100 placed in a 250 m*l* stoppered centrifuge bottle and then the bottle was continuously shaken at 30°C on a reciprocal shaker (amplitude 7 cm, 110 strokes/min). The cells that had taken up cadmium were collected by centrifugation (3,000×g, 10 min), washed 5 times with distilled water and dried at 105°C for 18 hours. Amounts of cadmium (mg/g of dry cells) taken up by the living cells almost linearly decreased with the increments of the concentration of the cells. Whereas the amounts of cadmium (mg) taken up by the cells increased progressively up to the cell concentration of 0.15g/100 m*l* of saline solution as dry cells (data not shown). In this respect, throughout this experiment cell concentration was adjusted to bellow 0.15g/100 m*l* as dry cells.

Washing of the cells with EDTA solution

Elution of cadmium taken up by the cells was performed by a modification of Nakajima's method (11). The living or scalded cells that had taken up cadmium were suspended in 0.01 M or 0.1 M EDTA solution by stirring gently for a designated time at room temperature. After being in contact with the EDTA solution, the cells were collected by centrifugation. The same washing process was repeated 3 times and then the cells were thoroughly with deionzed water. The cadmium eluted by the 3 times washing with 0.01 M EDTA solution was defined as physicochemical adsorption onto cell surface, whereas the cadmium remaining in the cells after washing with EDTA solution was defined as intracellular accumulation by biological activity.

Treatment of the cells with metabolic inhibitor

The yeast cells were suspended in 100 m*l* of saline solution containing 100 μg/m*l* of cadmium and 10^{-3} or 10^{-4} M of each metabolic inhibitor by shaking at room temperature. After 24 hours contact with the metabolic inhibitors, the cells were collected, washed thoroughly with distilled water and then used for the cadmium determination.

Determination of cadmium

As described in the previous paper (7), cadmium was determined by atomic absorption method using an atomic absorption spectrophotometer (Shimadzu AA-646) with 228.8 *nm* wavelength.

Chemicals

The standard solution of cadmium (1,000 μg/m*l*) and ethylenediamintetraacetic acid, disodium salt (EDTA) were purchased from Hayashi Pure Chemicals Co., Osaka, Japan. Triton X-100, 2,4-dinitrophenol

(DNP), sodium azide and sodium cyanide were obtained from Wako Pure Chemicals Co., Tokyo, Japan. The polypeptone and yeast extract used were obtained from Sigma Chemicals Co., St. Louis, Missouri, U.S.A. and the other regents used guaranteed ones.

Results and Discussion

Effect of cadmium ion on the cadmium uptake

The cadmium uptake by the living or the scalded cells was studied. As shown in Table 1, the amounts of cadmiun taken up increased progressively in proportion to increment of cadmium concentration in living cells and scalded cells of cadmium tolerant yeast. And the amounts of cadmium taken up by the scalded yeast cells were 3 times more than the value of the living cells in a low concentration of cadmium, and in a higher concentration of cadmium (100 μg/m*l*) were 2 times more.

On the other hand, uptake efficiency of cadmium adversely decreased in proportion to the increments of cadmium concentration and the uptake efficiency of cadmium of the scalded cells was 3 times above that of the living cells.

These results were similar to the uptake of manganese (11) and uranium (12) by *Chlorella regularis,* and furthermore to the adsorption of cadmium (13) by many bacterial cells. In the scalded *Chlorella regularis* (11), the uptake capacity of metals was remarkably increased. It was concluded that the uptake of *Hansenula anomala* B-7 was independent of the biological activity but dependent on the increment of the capacity of the physical adsorption due to the denaturation of the cell surface by heat treatment.

Distribution of cadmium taken up

To investigate the distribution of cadmium taken up into the cadmium tolerant yeast cell, the intact cells were suspended in distilled water containing 100 μg/m*l* of cadmium for various cultivation times and then the cadmium adsorbed onto the outer layer of cell was eluted by washing with 0.01 M of EDTA solution 3 times.

As shown in Table 2, in the living cells the amounts of cadmium adsorbed or accumulated were increased in proportion to the extent of exposure time, but in the scalded cells the amount of cadmium adsorbed was not affected by exposure time and cadmium was scarcely accumulated by the cells. Furthermore, when the living cells were incubated in 100 μg/m*l* of cadmium for 20 hours 74% of the cadmium taken up was adsorbed onto the outer layer of living *Hansenula anomala* B-7 cells and 26% of it was accumulated into the cells, whereas the scalded cells adsorbed 98.3% of the cadmium taken up and accumulated only 1.7% of it into the cells.

A cupric acetate-tolerant yeast, *Candida sake* A, adsorbed 39.2% of Cu^{2+} incorporated into the cells, and 35.8%, 16.0% and 9.0% of it were accumulated in the intracellular soluble fraction, cold acid soluble fraction and hot acid soluble fraction, respectively (14).

The adsorption *vs.* accumulation ratio by the living cells in this study was different from those of cupric acetate-tolerant yeast. The adsorption *vs.* accumulation ratio of cadmium taken up by the living or scalded cells showed the similar values in the different cocentration of cadmium (data not shown).

Effect of Triton X-100

The uptake efficiently of cadmium by the intact

Table 1. Effect of cadmium concentration in cell suspension on cadmium uptake.

Cadmium concentration (μg/m*l*)	Living cells		Scalded cells	
	Cadmium taken up (mg)	Uptake efficiency (%)**	Cadmium taken up (mg)	Uptake efficiency (%)**
10	0.122 (100)*	12.4	0.365 (299)	36.0
50	0.224 (100)	4.5	0.683 (304)	14.0
100	0.342 (100)	3.4	0.735 (214)	7.0

*Denotes relative value based on living cells.

**Uptake efficiency $= \frac{\text{Total cadmium in cells (mg)}}{\text{Cadmium in cell suspension (mg)}} \times 100$

The uptake of cadmium was carried out in cell suspensions containing 100 μg/m*l*. of cadmium plus 0.1% of Triton X-100 at 30°C for 24 hours on a reciprocal shaker.

Table 2. Distribution of cadmium taken up by the living and scalded cells.

	Exposure time (hr.)	Adsorbed cadmium (mg)	Accumulated cadmium (mg)	Adsorbed Cd^{+2} *vs* Accumulated Cd^{+2} (%: %)
Living	1	0.106	0.028	79.1: 20.9
cells	4	0.221	0.080	73.4:26.6
	20	0.358	0.126	74.0:26.0
Scalded	1	0.738	0.011	98.5:1.5
cells	4	0.754	0.011	98.6:1.4
	20	0.772	0.013	98.3:1.7

Table 3. Effect of Triton X-100 on uptake or accumulation of cadmium.

		Cadmium taken up (mg/g of dry cells)	Cadmium accumulated (mg/g of dry cells)
Living	None	2.65 (100)*	0.171 (100)*
cells	Triton X-100	4.30 (162.3)	0.471 (275.4)
Scalded	None	7.64 (100)*	
cells	Triton X-100	7.51 (98.3)	

*: Denotes relative value (%) based on absence of Triton X-100.

cells was enhanced up to approximately 40% or more by both 0.1% of Triton X-100 and Aerosol OT (10). The effect of Triton X-100, a non-ionic surfactant, on the uptake and intracellular accumulation of cadmium was examined in the living and scalded cells. As shown in Table 3, a cadmium uptake (adsorption plus accumulation) by the living cells was accelerated up to 162.3% compared to surfactant-free, but the amount of cadmium taken up by the scalded cells was the same regardless of Triton X-100. The intracellular accumulation of cadmium was accelerated up to 275.4% by Triton X-100 in the living cells compared with surfactant-free.

These results indicated that Triton X-100 increased biological activity of the living cells and then accelerated uptake of cadmium. Furthermore, in the scalded cells Triton X-100 did not affected the uptake of cadmium and this result suggested that the uptake of cadmium by the scalded cells depended upon the physicochemical adsorption on the cell surface and not upon the biological activity.

Release of cadmium by washing with EDTA

The distribution and the uptake states of cadmium were examined by washing with EDTA solution in the living or the scalded *Hansenula anomala* B-7 cells. As shown in Table 4, 58.0% of the cadmium taken up was released from the living cells by washing with 0.1 M EDTA solution for 5 minutes and 67.5% of it was released after 60 minutes. But 93.3% and 96.7% of the cadmium taken up were released from the scalded cells by washing with EDTA solution for 5 and 60 minutes, respectively. A slight amount of the cadmium taken up by the living cells was released in the washing process with 0.1 M solution, whereas most of the cadmium taken up by the scalded cells was rapidly released by washing with EDTA solution. These results were very similar to the release ratios of uranium (12) and manganese (11) from the living and the scalded *Chlorella regularis* cells.

This far more rapid release of cadmium from the scalded cells by washing with EDTA solution in contrast to the living cells suggested that in the living cells cadmium was incorporation into the inner part of cells by the biological activity. On the other hand, in the scalded cells cadmium was adsorbed onto the cell surface and thus coupled with the ligands which were able to be easily substituted with EDTA solution. This result suggested that the cadmium uptake by the scalded *Hansenula anomala* B-7 cells was almost completed by the physico-chemical adsorption onto the cell surface.

Effect of metabolic inhibitors

To examine whether this larger uptake of cadmium by the living cells depends on the physico-chemical adsorption on the cells surface or on the biological activity, the effects of metabolic inhibitors on the cadmium uptake by the living or the scalded cells were tested.

Table 5 showed that the cadmium uptake by the living *Hansenula anomala* B-7 cells was inhibited by uncouplers of oxidative phosphorylation like 2,4-dinitrophenol (DNP) and inhibitors of the electron transport like sodium azide (NaN_3) wherease the scalded cells were not inhibited by metabolic inhibitors used in this study. Furthermore, metabolic inhibitors caused inhibition of cadmium uptake or its accumulation by the livings cells. The degree of inhibition of cadmium uptake or accumulation was proportional to the concentration of metabolic inhibitor and was related to the biochemical properties of the inhibitors. And the inhibition rate of intracellular accumulation of cadmium by the metabolic inhibitors was larger than that of cadmium uptake (adsorption plus accumulation) under the same experimental conditions. On the other hand, the effect of metabolic inhibitors on the growth of *Hansenula anomala* B-7 and cadmium uptake by the strain during cultivation showed that both growth and cadmium uptake were greatly inhibited by any inhibitor including DNP, KCN and NaN_3 (data not shown). These results suggested that the accumulation of cadmium by the living cells was apparently dependent on cellular biological activity and also indicated evidence of the existance of an energy-dependent uptake system.

The aforementioned data suggested that intracellular accumulation of cadmium appeared to be the results of energy-requiring system, which was almost in accord with former results (3, 15).

Effect of shaking

The effect of shaking on cadmium uptake and intracellular accumulation by the living cells was investigated.

The amount of cadmium taken up was not affected by shaking and shaking rate, whereas the amounts of cadmium accumulated was slightly increased by shaking, and moreover its accumulation was significantly activated with the increment of shaking rate (data not shown).

Table 4. Release of cadmium taken up from yeast cells by washing with EDTA solution.

Washing time (min.)	Cadmium taken up (mg/g of dry cells)	
	Living cells	Scalded cells
0	2.674 (0)*	6.892 (0)*
5	1.122 (58.0)	0.462 (93.3)
60	0.869 (67.5)	0.229 (96.7)

*; Release ratio (%)

요 약

본 논문은 카드뮴 내성 효모, *Hansenula anomala* B-7에 있어서 카드뮴의 세포내 축적의 기작에 대하여 연구했다.

생물학적 활성을 상실시킨 scalded cells은 living cells보다 2~3배량의 카드뮴을 uptake 하였다. Living

Table 5. Effect of metabolic inhibitors on uptake or accumulation of cadmium.

	Inhibitor (0.1 mM)	Cadmium taken up (mg/g of dry cells)	Cadmium accumulated (mg/g of dry cells)
Living cells	None	5.32 (100.0)**	0.709 (100.0)**
	DNP*	4.20 (80.3)	0.511 (72.1)
	NaN_3	3.52 (67.3)	0.419 (59.1)
	KCN	–	0.431 (60.7)
Scalded cells	None	8.91 (100.0)**	–
	DNP*	8.75 (98.2)	–
	NaN_3	9.06 (101.6)	–

*; 2,4-dinitrophenol
**: Denotes relative value (%) based on the absence of metabolic inhibitors.

cells은 uptake한 카드뮴의 74%를 세포외층에 흡착시켰으며, 26%의 카드뮴은 세포내에 축적되었다. 그러나 scalded cells에서는 98.3%의 카드뮴을 세포외층에 흡착시켰으며, 1.7%의 카드뮴만을 세포내에 축적시켰다. Living cells은 Triton X-100에 의하여 카드뮴의 uptake와 축적이 162.3%와 275.4%씩 촉진되었으나, scalded cells은 아무런 영향을 받지 않았다. 더욱이 living cells은 카드뮴의 uptake와 축적이 대사저해제인 2,4-dinitrophenol, sodium azide 및 potassium cyanide에 의하여 강하게 저해되었으나, scalded cells에 있어서 카드뮴의 uptake는 아무런 영향을 받지 않았다.

이상의 결과로 카드뮴 내성 효모에 의한 카드뮴의 세포내 축적은 생물학적 활성에 의하여 이루어지며, 특히 전자전달계의 저해제인 sodium azide에 의하여 카드뮴의 축적이 강하게 저해되므로 에너지 의존성임을 시사했다.

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