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Effect of Cadmium on Protein Synthesis of Cadmium-Ion Tolerant *Hansenula anomala* B-7

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카드뮴 내성 *Hansenula anomala* B-7의 단백질 합성에 미치는 카드뮴의 영향

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In this study the authors investigated the distribution of cadmium accumulated in cadmium-ion tolerant *Hansenula anomala* B-7 cells and also the effect of cadmium on protein synthesis. 84.9% of the cadmium accumulated was distributed in the soluble fraction (cytosol, etc.). The intracellular protein content was decreased by cadmium (1,000 μg/m*l*), but the content of soluble protein precipitated by ammonium sulfate (30-75% saturation) was increased compared with the content of it obtained from the cells grown without cadmium. Furthermore, in the cells grown with 1,000 μg/m*l* of cadmium the higher molecular weight soluble protein was increased compared with the cells grown without cadmium, but the lower molecular weight soluble protein was decreased. These results suggested that the protein synthesis was inhibited by cadmium, but synthesis of higher molecular weight soluble protein was remarkably stimulated by cadmium.

In the previous papers, the authors have reported that *Hansenula anomala* B-7 cells took up a large amount of cadmium by Triton X-100 (1, 2) and seventy four percent of cadmium taken up by the living cells was adsorbed on the other layer of the cells and 26% of it was accumulated in the cells. Furthermore, the authors also suggested that an intracellular accumulation of cadmium apparently depended upon energy-dependent active transport system in these yeast cells (3).

Horitsu *et al.* (4) reported that about 88% of cadmium taken up was found in the cytoplasm of *Pseudomonas aeruginosa,* and approximately 80% of cadmium was present in the cytoplasm of *Escherichia coli* (5). Yu *et al.* (6) also reported that about 73% of cadmium taken up was found in the cytoplasm of *Hansenula anomala*B-7. However Kim *et al.* (7) observed that approximately 37% of cadmium accumulated in cells was disributed in the cytoplasm of *Enterobacter cloacae.* Their results are contradictory. In this work, as the first step for the investigation of the mechanism of tolerance to cadmium by the yeast cells the distribution of cadmium accumulated in the cells was examined. In addition, the electrophoretic pattern and ammonium sulfate fractionation pattern of soluble protein obtained from the cells cultivated with (1,000 μg/m*l*) or without cadmium and the change of protein content by cadmium were also investigated.

Key words: *Hansenula anomala* B-7, Intracellular distribution of cadmium, Effect of cadmium on protein synthesis.
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Materials and Methods

Unless otherwise noted, the materials and methods

were the same as described in the preceding papers (1, 2).

Fractionation of the yeast cells

Hansenula anomala B-7 was cultivated in the basal medium (3) containing 1,000 μg/m*l* of cadmium at 30°C for 24 hours. The yeast cells were harvested by centrifugation, washed 3 times with deionized water and finally washed 3 times with 0.01 M EDTA solution. Cadmium eluted from the cells by EDTA washing was considered as the adsorption fraction. In order to examine intracellular distribution, the EDTA-washing cells were suspended in distilled water and disrupted with a sonicator (120 Hz, 300 min) with cooling. The disrupted cells were centrifuged at 4,000×g for 10 min and the supernatant was recentrifuged at 16,000×g for 30 min, and then the cadmium in each fraction was determined.

Preparation of sample for electrophoresis and fractionation of ammonium sulfate

The havested cells were washed with saline solution, suspended in distilled water and disrupted with a sonicator (120 Hz, 30 min). The disintegrated cells were centrifuged at 8,000×g for 20 min and the supernatant was saturated with 30% ammonium sulfate at 4°C. After standing for 3 hours the precipitate was removed by centrifugation at 8,000×g for 20 min and discarded. The ammonium sulfate concentration was then increased to 75% by the addition of ammonium sulfate. After standing for 5 hours the resulting precipitate was collected by centrifugation at 16,000×g for 30 min and then dissolved in 0.01 M potassium phosphate buffer (pH 7.0). The suspension was dialyzed overnight against the same buffer.

The suspension dialyzed was used as sample for electrophoretic analysis.

Electrophoresis

Polyacylamide gel disc electrophoresis was performed by a modification of the Davis method (8). Stacking and running gels were polymerized in a test tube (6×100 mm). After run with a constant current of 8 mÅ per gel, it was stained with 1% Amido black 10B (E. Merck. Darmstadt, F.R.G.) and destained and stored in 7% acetic acid. Electrophoresis in the presence of sodium dodecyl sulfate (SDS) was performed according to the Weber and Osborn's method (9) in 5% gel with a normal amount of cross-linker 8 mÅ per gel. After the run the gel was stained with Coomassie brilliant blue R-250, destained and stored in 7% acetic acid. Sample protein was charged 100 μg or 200 μg in the presence or absence of SDS, respectively the electrophoretic pattern of gels was further investigated by using a densitometer (Cliniscan 2, Helena Laboratories, USA) at 525 *nm*.

Analysis

The soluble protein was determined by the method of Lowry *et al.* (10) with bovine albumin as a standard protein and the protein in whole cells was assayed by the Biuret method (11) with the same standard protein. Cadmium content was determined by the atomic adsorption method as shown in the previous paper (2).

Chemicals

Acrylamide was purchased from Wako Pure Chemicals Co., Tokyo, Japan. Methylene bisacrylamide, Coomassie brilliant blue R-250 and N,N,N,N'-tetramethylene diamine were obtained from Sigma Chemicals Co., St. Louis, Missouri, U.S.A. And all other chemicals used were guaranteed grade.

Results and Discussion

Intracellular distribution of cadmium

Intracellular distribution of cadmium in the *Hansenula anomala* B-7 cell was examined. The cells which were cultivated in the basal medium containing 100 μg/m*l* of cadmium under the conditions such as in the previous paper (2) were havested, washed with saline solution, and then finally washed with 0.01 M EDTA solution. The EDTA-washed cells were disrupted with a sonicator. In order to check the distribution of cadmium accumulated in the cells, the fractionation was carried out as shown in Table 1. About 8.0% of the cadimum accumulated was found in the precipitation fraction at 4,000×g for 10 min and was considered as mainly cell debris, etc. About 7.1% of the cadmium accumulated was distributed in the precipitate fraction at 16,000×g for 30 min and 84.9% of it was distributed in the supernatant fraction at the above conditions. It was concluded that the precipitate consisted of mitochondria and polysome etc., and that the supernatant consisted of microsome, ribosome and cytosol etc. These results were similar to the results of *Chlorella regularis* cells (12). The fact that most of the cadmium accumulated in the cells were distributed

Table 1. Distribution of cadmium accumulated in the cells.

Fraction	Total cadmium (mg)	Ratio (%)
Adsorption*	0.2890	73.8
Accumulation	0.1025	26.2 (100)**
Ppt. obtained by centri. at 4,000×g, for 10 min.	0.0082	2.1 (8.0)
Ppt .obtained by centri. at 16,000×g, for 30 min.	0.0073	1.9 (7.1)
Super. obtained by centri. at 16,000×g, for 30 min.	0.0870	22.2 (84.9)

* The cadmium eluted by washing with twenty m*l* of 0.01 M EDTA solution defined as adsorption fraction.
**Values in parentheses are the distribution ratios to cadmium contents of accumulation fraction.

in intracellular soluble fraction showed the possibility of the presence of a metallothionein such as cadmium-binding protein as a means of detoxification of the cadmium. We have already studied the cadmium-binding protein of *Hansenula anomala* B-7 (not published).

Effect of cadmium on the protein synthesis

In order to examine the effect of cadmium on the intracellular protein synthesis by cadmium tolerant *Hansenula anomala* B-7, the protein content in the cells grown with or without cadmium was determined.

As shown in Table 2, protein content was slightly decreased by cadmium, furthermore it was remarkably decreased by cadmium plus Triton X-100. This result suggested that cellular protein synthesis was inhibited by cadmium. Moreover, protein synthesis was strongly inhibited by cadmium plus Triton X-100.

It is concluded that Triton X-100 activated the permeability of extracellular cadmium into the cells, a concentration of intracellular cadmium ascended, and then protein synthesis was remarkably inhibited by a high concentration of intracellular cadmium.

Ammonium sulfate fractionation of soluble protein

As in the above results, protein synthesis was inhibited by cadmium. In order to study the precipitate patterns of soluble protein by ammonium sulfate, the soluble protein content obtained from the yeast cells grown with (1,000 μg/m*l*) or without cadmium was

Table 2. Comparison of protein content in the cells grown with or without cadmium.

Cultivated condition	Protein content* in the cells (mg/g of dry cells)
Cadmium free	44.2 (100)**
With cadmium (1,000 μg/m*l*)	36.7 (83.0)
With cadmium (1,000 μg/m*l*) plus 0.1% Triton X-100	22.0 (49.8)

*Assayed by Biuret method.
**Relative value based on the cells grown without cadmium.

adjusted to approximately 4 mg/m*l* respectively and then the soluble protein was fractionated by ammonium sulfate.

As shown in Table 3, by ammonium sulfate fractionation the protein content of the precipitate (0-30% saturation) and of the supernatant (30-75% saturation) obtained from the cells grown with cadmium was less than that of protein obtained from the cells grown without cadmium under the same conditions. On the order hand, by ammonium sulfate fractionation (30-75% saturation) the content of soluble protein obtained from the cells grown with cadmium was conversely more than that of the protein obtained from the cells grown without cadmium.

As the results above described, it was concluded that changes in the precipitate pattern of soluble protein by ammonium sulfate were related to those of the solubility of protein obtained from the cells grown with cadmium (1,000 μg/m*l*).

Comparison of electrophoretic pattern of soluble protein

The variation of soluble protein from the tolerant yeast cells grown with or without cadmium was investigated by disc-and SDS-electrophoretic analysis.

As shown in Fig. 1, the patterns of soluble protein by disc-and SDS-electrophoresis were similar but the patterns from cells cultivated with or without cadmium showed significant differences. In the cells grown with 1,000 μg/m*l* of cadmium the higher molecular weight soluble protein content was more than in the cells grown without cadmium, furthermore, the lower molecular weight soluble protein content was less than in cadmium free conditions, as shown in Fig. 1 (A).

Horitsu *et al.* reported that a difference was

Table 3. Ammonium sulfate fractionation of solble protein.

Cultivated condition	0-30% $(NH_4)_2SO_4$	30-75% $(NH_4)_2SO_4$		Total protein (mg)
	Precipitate (mg)	Precipitate (mg)	Supernatant (mg)	
Cells grown without cadmium	18.75 (17.51)	46.36 (43.30)	41.95 (39.18)	107.06 (100)*
Cells grown with 1,000 μg/m*l* of cadmium	17.36 (15.76)	54.21 (49.22)	38.57 (35.02)	110.14 (100)

*Denotes relative value based on the cells cultivated without cadmium.
Protein content in both of the soluble protein fractions obtained from the yeast cells cultivated with (1,000 μg/m*l*) or without cadmium before ammonium sulfate fractionation was ca. 4 mg/m*l*.

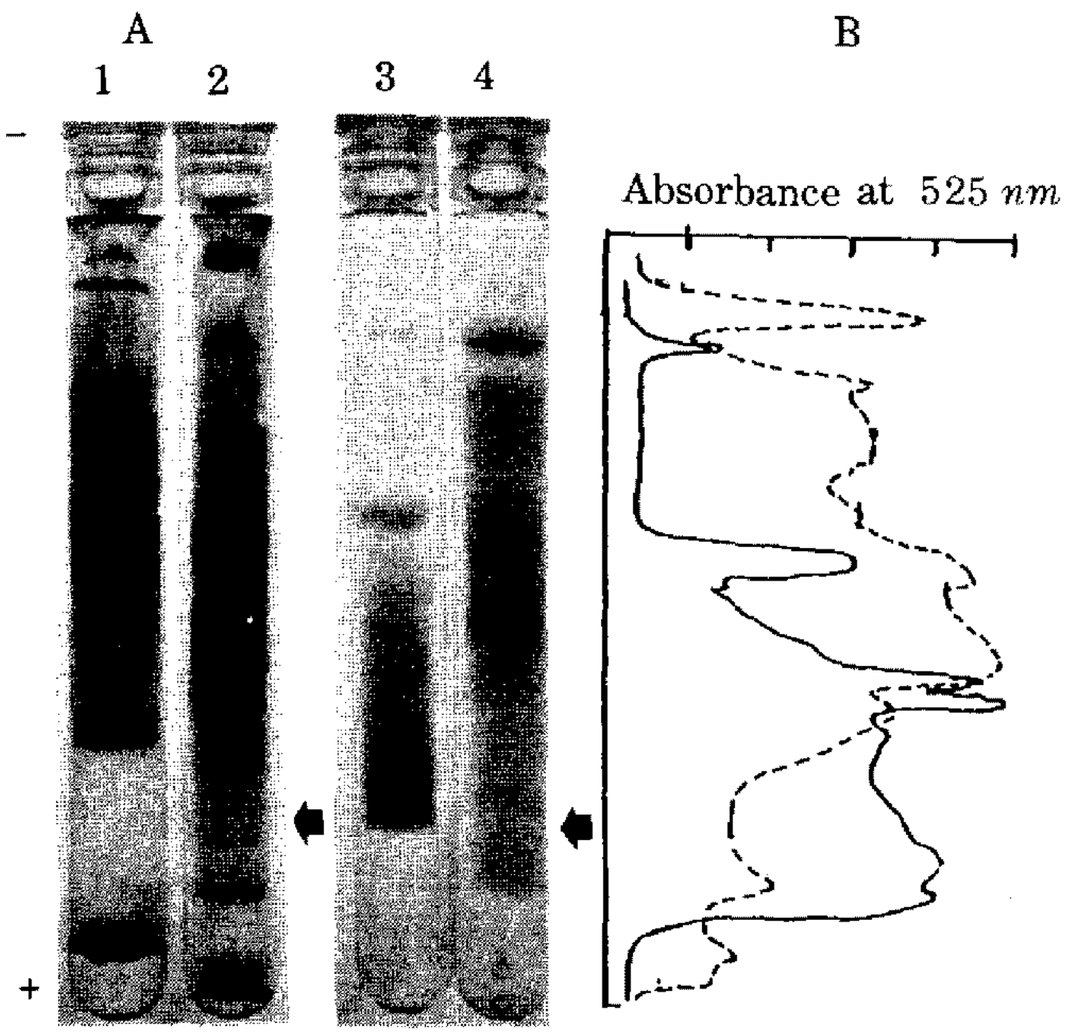

Fig. 1. Electrophoretic patterns (A) in the absence (lane 1 and 2) or presence (lane 3 and 4) of sodium dodecyl sulfate, and densitograms (B) of lane 3 and 4.
Lane 1 and 3 were pattern of the soluble fraction from the cells cultivated without cadmium, and lane 2 and 4 were pattern of it with cadmium. Detailed conditions are described in Materials and Methods.
—— , without cadmium; ------ , with cadmium.

observed between disc electrophoretic patterns; some proteins were detected only in the preparation of cadmium-grown *Pseudomonas aeruginosa* cells, and the cadmium-affected protein was located in the anode region of disc electrophoretic pattern. As shown in Fig. 1 (A), the characteristic protein band (arrow mark) was not observed in the soluble protein fraction from the cadmium tolerant yeast cells grown without cadmium, whereas it was observed in the soluble protein fraction from the cells grown with 1,000 μg/m*l* of cadmium under the same conditions. Moreover, the characteristic protein band (arrow mark) was located in the anode region of the disc-and SDS-electrophoretic pattern and the protein was a lower molecular weight. Their results were similar to the those of Horitsu *et al* (4).

Pigment formation

In this study, yellowish pigment formation was occasionally observed by cultivating the cadmium tolerant yeast in the presence of 1,000 μg/m*l* of cadmium and in the presence of 1,000 μg/m*l* of cadmium plus 0.1% of Triton X-100. The visible spectrum of the pigment had its maximum value at 360 *nm*. The cells were cultivated in basal medium containing 1,000 μg/m*l* of cadmium plus 0.1% of Triton X-100 under standard conditions, the cells were removed by centrifugation, and a culture fluid was obtained. With basal medium as a blank, the value of optical density of the culture fluid at 360 *nm* was 2.11 (data not shown).

The factors and the conditions of pigment formation were not known, so further study is required.

요 약

카드뮴 내성 효모, *Hansenula anomala* B-7 세포에 축적된 카드뮴의 세포내 분포와 단백질 합성에 미치는 카드뮴의 영향 등에 대하여 연구했다.

세포내 축적된 카드뮴의 84.9%는 cytosol 등인 가용성 분획에 존재했다. 세포내 단백질 함량은 카드뮴(1,000 μg/m*l*)에 의하여 감소되었으나, 유안(30~75% 포화)에 의하여 침전되는 가용성 단백질의 함량은 카드뮴에 의하여 증가되었다. 더욱이 카드뮴(1,000 μg/m*l*)의 첨가배지에서 배양된 세포에는 고분자 가용성 단백질

이 카드뮴 무첨가 배지에서 배양된 세포에서 보다 증가되었으나, 저분자 단백질은 감소되었다.

이상의 결과로 단백질의 합성은 카드뮴에 의하여 저해되나, 유안(30~75% 포화)에 의하여 침전되는 고분자 단백질의 합성은 카드뮴에 의하여 촉진된다.

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