KOR. JOUR. MICROBIOL. September, 1990 p. 258-263 Vol. 28, No. 3

Purification and Characteristics of Cadmium-Binding Protein from *Hansenula anomala*

Yu, Tae-Shick and Bon-Kyung Koo
Department of Microbiology, College of Natural Science, Keimyung University, Taegu, Korea

*Hansenula anomala*이 생성하는 Cadmium-Binding Protein의 정제 및 특성

유대식 · 구본경
계명대학교 자연과학대학 미생물학과

ABSTRACT: A cadmium-binding protein was purified the cell-free extract of extreme cadmium tolerant *Hansenula anomala* B-7. The molecular weight was determined to be approximately 33,000 and was composed two kinds of subunits having a molecular weight of 18,000 and 14,000, respectively.

The extinction coefficient $E^{1\%}_{280\ nm}$ of the cadmium-binding protein was calculated to be 19.58.

The amount of cadmium in the cadmium-binding protein was 9.26 μg per 100 μg of protein.

A total of 14 amino acids were detected in the cadmium-binding protein, including aspartic acid, glycine and alanine that were present in a high quantity, but proline, valine and methionine were not found.

The purified cadmium-binding protein contained a high quantity of cysteine and cadmium, and therefore this protein showed clearly the characteristics of metallothionein.

KEY WORDS □ **Cadmium-binding protein, *Hansenula anomala* B-7**

Organisms, especially microorganisms are known to employ a large variety of mechanisms for adaptation to the presence of heavy metal ions (Gadd *et al.* 1978; Aiking *et al.* 1984). An interesting adaptation mechanism was described by Mitra *et al.* (1975) and Yu *et al.* (1986). Mitra *et al.* (1975) reported that cells of *Escherichia coli* strain B developed larger intracellular vacuoles and exhibited an abnormally long lag phase in a defined medium containing a high concentration of cadmium ion. An extreme cadmium tolerent *Hansenula anomala* B-7 showed that in presence of 1,000 μg/m*l* of cadmium the lag phase of this strain was lengthened compared with conditons without cadmium (Yu *et al.* 1986). For adaptation to the presence of heavy metals many strains are stimulated to induce the synthesis of a specific metal-binding protein which was very different from metallothioneins and is considered to be a protein for homeostasis and/or detoxification of heavy metals. (Murasugi *et al.* 1981).

Metallothionein is the metal-binding protein that is induced by heavy metals, and it contains large amounts of bound metals and cysteine residues. They were found in soybeans (Yoshida, 1986), rice plants (Kaneta *et al.* 1983), blue-green alga (Olafson *et al.* 1979) *Tetrahymena pyrisormis* (Nakamura *et al.* 1981) and *Schizosaccharomyces pombe* (Murasugi *et al.* 1981).

In the present paper, the authors reported that in the *Hansenula anomala* B-7 cells grown with 1,000 ug/m*l* of cadmium the characteristic protein bands were located in the anode region of the disc- and SDS-electrophoretic patterns and the protein was a lower molecular weight. We surmised that this protein was a specific cadmium-binding protein (Yu and Song, 1990).

In this paper, the purification and some properties of cadmium-binding protein from extreme cadmium

tolerant *Hansenula anomala* B-7 are described.

Materials and Methods

Microorganism

Hansenula anomala B-7 that was isolated by the present authors (Yu and Song, 1981; Yu *et al.* 1986) as an extreme cadmium tolerant yeast was used throughout this study.

Media and culture conditions

The medium contained 10g of glucose, 10g of polypeptone, 5g of yeast extract, 1g of NaCl, 0.3g of $MgSO_4 \cdot 7H_2O$, and 0.1 g of KH_2PO_4 in 1,000 m/ of deionized water, and the pH was adjusted to 6.0. The yeast cells were grown in 4 L of medium containing 1,000 μg/m/ of cadmium in 5 L jar-fermentor at 28 °C for 6 days. The medium contained 0.1% Triton X-100 as an activator of cadmium accumulation, and Silicone KM-70 was added as an antiformer.

The cells were harvested by centrifugation at 5,000 × g for 10 min, and were washed with 0.9% NaCl solution twice. The growth was determined by measuring the optical density of the culture at 660 *nm* and dried cell weight (g).

Preparation of cell-free extract

The washed cells were suspended in 0.01 M Tris-HCl buffer (pH 7.0), and disintegrated portion by portion in a glass-bead mill (Bead-Beater; Biospec Products, Bartlesville, Okla.) with a 350 m/ removable blending chamber with ports opened at the top. When used, the chamber contained about 230 m/ of 0.3 mm glass beads, and was cooled to below 10 °C with ice-water circulating in its outer jacket.

Milling was monitored by occasional inspections under a microscope of samples withdrawn through the ports, and was stopped when almost all cells had been disintegrated. The chamber was removed, and after the glass beads settled, the mixture was replaced with another portion of the cell suspension through the ports by decantation. The milling was then done for the same period as before. The mixtures were pooled, combined with the washings of the glass beads, and centrifuged to remove cell debris at 9,000 × g for 30 min. The resultant clear solution was referred to as the cell-free extract.

Electrophoresis

Polyacrylamide gel electrophoresis was performed by a modification of Davis' method (1964). Stacking and running gels were ploymerized in a test tube (5 × 65 mm). After running with a constant current of 8 mA per gel, the gel was stained with 1% Amido black 10 B (E. Merck, Darmstadt), electrophoretically 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 (1969) in 5% gel with the normal amount of cross-linker at 5 mA per gel. After running, the gel was stained with Coomassie brilliant blue R-250 (Sigma), electrophoretically destained, and stored in 7% acetic acid.

Molecular weight

The molecular of the cadmium-binding protein was estimated by gel filtration according to the method described by Andrews (1965). Gel filtration was performed in a Ultrogel A-6 column (1.8 × 85 cm) previously equilibrated with 0.01 M Tris-HCl buffer (pH 7.0). The purified cadmium-binding protein and standard proteins were applied onto the column and 3 m/ fractions were collected. The standard proteins used for calibration were as follows; ferritin (Mr. 443,000), alcohol dehydrogenase (Mr. 150,000), bovine serum albumin (Mr. 68,000) and ovalbumin (Mr. 45,000). SDS-polyacrylamide gel electrophoresis was also employed for the determination of the molecular weight of subunit of the cadmium-binding protein according to the Weber and Osborn's method (1969). The standard proteins that were used were from a kit of standard proteins of low molecular weight; namely, rabbit muscle phosphorylase b (Mr. 94,000), bovine serum albumin (Mr. 67,000), ovalbumin (Mr. 43,000), bovine erythrocyte carbonic anhydrase (Mr. 30,000), soybean trypsin inhibitor (Mr. 20,100) and bovine milk α-lactalbumin (Mr. 14,400).

Amino acid analysis

The amino acid composition was determined by the method of Spackman *et al.* (1958). A four milligrams of the lyophilized cadmium-binding protein were hydrolyzed in duplicate with 4 m/ of 6N HCl at 110 °C for 24 hr in sealed tubes *in vacuo*. Amino acid analysis of the hydrolyzates was carried out with a LKB 4150 ALPHA amino acid analyzer (Sweden).

Cadmium analysis

The cadmium was analyzed by atomic absorption method using atomic absorption spectrophotometer (Schimadzu AA-646) at 2,288A as shown in the previous paper (Yu *et al.* 1987).

Protein determination

Protein was determined by the method of Lowry *et al.* (1951) with ovalbumin as a standard protein and was measured spectrophotometrically at 280 *nm*.

Chemicals

Cadmium nitrate and the standard solution of cadmium were purchased from Hayashi Chemicals Co., Osaka. Triton X-100, Silicone KM-70 and a kit

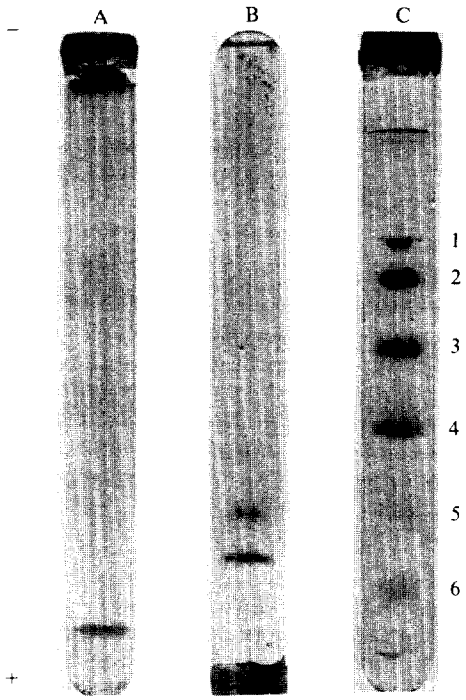

Fig. 1. *Polyacrylamide gel electrophoresis in the absence (A) and presence (B and C) of sodium dodecyl sulfate.*
Lanes A and B, purified cadmium-binding protein, Lane C, standard proteins.

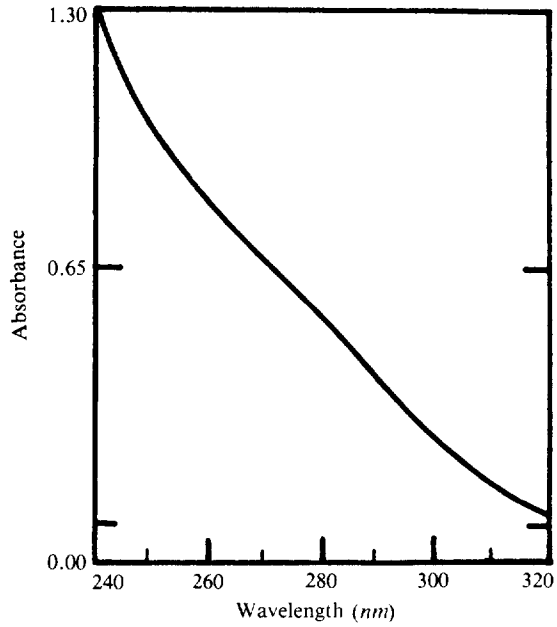

Fig. 2. *Ultraviolet absorption spectrum of a cadmium-binding protein.*
Ultraviolet absorption of a cadmium-binding protein (0.63 ug/ml) from *Hansenula anomala* B-7 was measured in 0.01M Tris-HCl buffer (pH = 7.0) at room temperature.

of low molecular weight standard for electrophoresis were purchased from Wako Pure Chemicals Co., Tokyo, Shinetsu Chemicals Co., Osaka, and Pharmacia KK., Tokyo, respectively. Ultrogel AcA 44, A-6 and A-2 were obtained from IBF Pharmindustrie, Villeneuve-la-Garenne, France.

RESULTS AND DISCUSSION

Purification of the cadmium-binding protein

All purification steps were carried out at 5°C unless otherwise noted.

Step. 1. Ammonium sulfate fractionation.

To the cell-free extract solid ammonium sulfate was added to give 30% saturation and the pH was adjusted 7.0. After standing for 4 hr, the resulting precipitate was removed by centrifugation at 9,000 × g for 20 min and discarded. Solid ammonium sulfate was added to the supernatant to give 70% saturation and allowed to stand for 4 hr. The resulting precipitate was collected in the same manner and dissolved in 0.01 M Tris-HCl buffer (pH 7.0).

Step 2. Ultrogel AcA 44 gel filtration

The concentrated protein solution was introduced into a Ultrogel AcA 44 column (2.7 × 85 cm) equilibrated with 0.01 M Tris-HCl buffer (pH 7.0). The buffer was allowed to flow at rate of 18 ml per hr and 5 ml fractions were collected. To the fractions containing cadmium, solid ammonium sulfate was added to give 70% saturation. After standing 4 hr, the precipitate was collected by centrifugation at 12,000 × g for 30 min and dissolved in the same buffer.

Step. 3. Ultrogel A-2 gel filtration.

The crude cadmium-binding protein solution was applied to a Ultrogel A-2 gel column (1.8 × 85 cm) equilibrated with the same buffer. The buffer was allowed to flow at a rate 18 ml per hr and 3 ml fractions were collected. Active fractions were combined and concentrated by pervaporation with a dialysis membrane.

Step. Ultrogel AcA 44 gel filtration.

The concentrated cadmium-binding protein solution was introduced to a Ultrogel AcA 44 gel column (1.8 × 85 cm) equilibrated with 0.01 M Tris-HCl buffer (pH 7.0). The buffer was allowed to flow at a rate of 10 ml per hr and 3 ml fractions were collected.

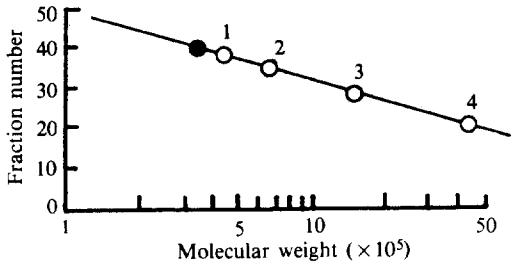

Fig. 3. *Molecular weight determination of a cadmium-binding protein by Ultrogel A-6 gel filtration.*
The standard proteins used and their molecular weights were: 1, ovalbumin (Mr. 45,000); 2, bovine serum albumin (Mr. 68,000); 3, alcohol dehydrogenase (Mr. 150,000); 4, ferritin (Mr. 443,000) Detailed descriptions are given in the Materials and Methods. ○, standard proteins; ●, cadmium-binding protein.

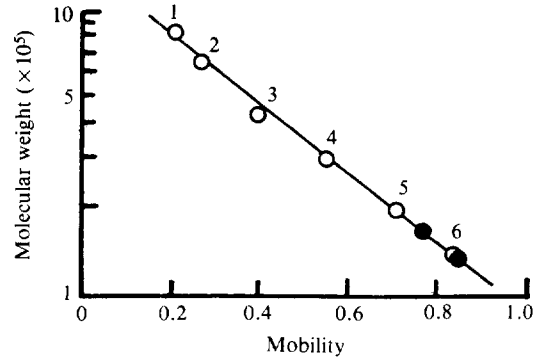

Fig. 4. *Molecular weight determination of subunit of a cadmium-binding protein by SDS-polyacrylamide gel electrophoresis.*
A cadmium-binding protein was located in the same gel as described in Fig. 1. A standard proteins were subjected to the electrophoresis in the same manner. The standard proteins and their molecular weights were: 1, rabbit muscle phosphorylase b (Mr. 94,000); 2, bovine serum albumin (Mr. 67,000); 3, ovalbumin (Mr. 43,000); 4, bovine erythrocyte carbonic anhydrase (Mr. 30,000); 5, soybean trypsin inhibitor (Mr. 20,100); 6, bovine milk α-lactalbumin (Mr. 14,400). Detailed descriptions are given in the Materials and Methods. ○, standard proteins; ●, cadmium-binding protein.

Homogeneity of the purified cadmium-binding protein

The purified cadmium-binding protein from *Hansenula anomala* B-7 was subjected to polyacrylamide gel electrophoretic analysis.

The cadmium-binding protein showed a single band on acrylamide gel electrophoresis in the absence of sodium dodecyl sulfate (SDS), and shown in Fig. 1. This result indicated that the purified protein was homogeneous. However, the protein showed two bands located in the anode zone on polyacrylamide gel electrophoresis in the presence of SDS; one band that strongly stained with Coomassie brilliant blue was located near the front of the gel (at R_f 0.85) and the other band weakly stained was located at R_f 0.77. Therefore the cadmium-binding protein consisted of two identical subunits.

UV absorption spectrum

As shwon in Fig. 2, the UV absorption spectrum of cadmium-binding protein had a high absorption at 280 *nm*. The UV absorption spectrum of protein showed generally a shoulder at 250 to 280 *nm*. But a shoulder of UV absorption spectrum of the purified cadmium-binding protein was almost disappeared. The UV absorption spetrum of this cadmium-binding protein indicated the specific characteristics of cadmium-thionein (Nakamura *et al.* 1981).

The UV absorption spectrum of this cadmium-binding protein is similar to those of a metal-binding protein of *Tetrahymena pyrisormis* (Nakamura *et al.* 1981) and a cadmium-binding protein of cadmium-treated rice plants (Kaneta *et al.* 1983).

The extinction coefficient $E^{1\%}_{280\ nm}$ of the cadmium-binding protein was calculated to be 19.58.

Cadmium content

There was 9.26 μg of the cadmium per 100 μg of the cadmium-binding protein (data not shown). The cadmium contained 3.7g atoms and 1.28g atoms per moles of the metal-binding protein from *Tetrahymena pyriformis* (Nakamura *et al.* 1981) and from *Synechococcus* sp. (Olafson *et al.* 1979), respectively.

The cadmium-binding protein of cadmium tolerant *Hansenula anomala* B-7 contained a higher content of cadmium than those of *Tetrahymena pyrisormis* and *Synechococcus* sp.

Molecular weight

The molecular weight of native cadmium-binding protein of cadnium tolerant *Hansenula anomala* B-7 was estimated to be about 33,000 by gel filtration according to the method described by Andrews (1965), as shown in Fig. 3. The molecular weight of a subunit of the protein was determined to be about 18,000 and 14,000 by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate according to the Weber and Osborn's method (1969), as shown in Fig. 4.

The molecular weights of the cadmium-binding

Table 1. *Amino acid composition of a cadmium-binding protein from Hansenula anomala B-7.*

No.	Amino acid	Amount of amino acid (n mol)	(% of total residues)
1	Asp-Asn	13.366	10.63
2	Thr	8.899	7.08
3	Ser	7.349	5.85
4	Glu-Gln	8.305	6.61
5	Pro	ND	ND
6	Gly	21.840	17.37
7	Ala	15.073	11.99
8	Cys	9.200	7.32
9	Val	ND	ND
10	Met	ND	ND
11	Ile	7.387	5.88
12	Leu	10.690	8.50
13	Tyr	3.545	2.82
14	Phe	0.487	0.39
15	His	8.914	7.09
16	Lys	10.000	7.95
17	Arg	0.675	0.54
Total amino acid amount		125.730 n mol	100.00%

Total amino acid amount per 50 u/ sample (4 mg protein/m/).
ND; not determined.

protein of *Escherichia coli* (Khazaeli and Mitra, 1981) and *Tetrahymena pyrisormis* (Nakamura *et al.* 1981) were 39,000 and 11,000, respectively. Furthermore, a molecular weight of cadmium-binding protein I and cadmium-binding protein II of *Schizosaccharomyces pombe*(Murasugi *et al.* 1981) were 4,000 and 1,800, respectively.

From the differences of molecular weight of the metal-binding protein, it was concluded that the cadmium-binding protein from cadmium tolerant *Hansenula anomala* B-7 was induced by cadmium, had two kinds of molecular forms, and bound cadmium in different forms.

Amino acid composition

The amino acid composition of cadmium-binding protein from *Hansenula anomala* B-7 is shown in Table 1. This protein showed 14 kinds of amino acids out of the 17 kinds of amino acids which can be determined with the amino acid analyzer used in this study.

This protein contained a high quantity of glycine (17.37%), alanine (11.99%) and aspartic acid-asparagine (10.63%).

Proline, valine and methionine which had been found in a metal-binding protein of *Tetrahymena pyrisormis* (Nakamura *et al.* 1981) and soybeans (Yoshida, 1986) were not determined. Generally, heavy metal-binding protein is rich in cysteine. A metal-binding protein of *Tetrahymena pyrisormis* contained a very high content of cysteine (32.4%) but the protein of soybeans from a cadmium polluted field contained a low content of cysteine (1.9%). The cadmium-binding protein of *Hansenula anomala* B-7 contained a 7.32% of cysteine of the total content of amino acids.

From the above results, the cysteine content of metal-binding protein is very contrary. Contents of acidic amino acids such as aspartic acid-asparagine, glutamic acid-glutamine were higher than those of basic amino acids such as histidine, lysine and arginine.

적 요

고도 카드뮴 내성 *Hansenula anomala* B-7으로 부터 카드뮴 결합 단백질을 분리하여 전기 영동적으로 단일단백질로 정제됨을 확인했다.

이 단백질의 분자량은 약 33,000으로서, 분자량 18,000과 14,000의 subunit로 구성되어 있다.

카드뮴 결합 단백질의 $E^{1\%}_{280nm}$는 19.58이였으며, 100 μg의 단백질 중에 9.26 μg의 카드뮴을 함유했다.

카드뮴 결합 단백질로 부터 14종류의 아미노산이 검출되었으며, aspartic acid, glycine과 alanine이 비교적 많이 함유되어 있으며, proline, valine 및 methionine은 검출되지 않았다.

본 연구에 사용된 카드뮴 내성효모로 부터 분리, 정제된 카드뮴 결합 단백질은 cysteine과 카드뮴을 다량 함유하여 metallothionein의 특징을 잘 나타내고 있었다.

사 사

본 연구는 1988년도 계명대학교 비사 교수연구기금으로 이루어졌음.

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(Received June 21, 1990)
(Accepted August 30, 1990)