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Identification and Partial Purification of Two Hydrogenase Isoenzymes from *Escherichia coli*

Choi, Suk-Jung and Chul-Hak Yang*

Dept. of Chemistry, Kangreung National Univ., Kangreung, Kangwondo 210-702, Korea,
**Dept. of Chemistry, Seoul National Univ., Seoul 151-742, Korea*

대장균으로부터 두 종류의 수소발생 동위효소의 확인과 부분정제

최석정 · 양철학*

강릉대학교 화학과
*서울대학교 화학과

ABSTRACT: The membrane-bound *Escherichia coli* hydrogenases were purified partially by the solubilization with detergents. The *E. coli* crude extract was solubilized with sodium deoxycholate and dialyzed against the buffer containing Triton X-100. Two different hydrogenases were obtained by the DEAE-cellulose, hydroxyapatite and Sephedex G-200 column chromatography. The one was unstable during purification and contained 70- and 47-kDa polypeptides as major proteins. The other showed high H2-evolving activity and had major polypeptides of Mr 31 and 27. Those polypeptides were detected by the two-dimensional electrophoresis.

KEY WORDS □ ***Escherichia coli*, hydrogenase isoenzymes, sodium deoxycholate, Triton X-100**

Introduction

The enzyme hydrogenase (E.C. class 1.12) catalyzes the production and the consumption of gaseous hydrogen in the presence of an appropriate electron carrier. It has been found in a diverse group of microorganisms, including both aerobic and anaerobic bacteria, as well as algae and protozoa and plays a major role in the metabolism of anaerobic bacteria. Hydrogenase of various organisms are different in physiological role, cellular location and oxygen sensitivity. (Adams *et al.*, 1981) But, they are all iron-sulfur proteins and some contain nickel or selenium. In many cases, the enzyme consists of two different subunits, a large one (60-70 kDa) and a small one (25-35 kDa). The purification of E.coli hydrogenases was reported by several groups. (Adams and Hall, 1979; Saweres and Boxer, 1986; Ballantine and Boxer, 1986; Stoker *et al.*, 1988) Immunologically different three isoenzymes were found and one of them was electrophoretically labile. However, their interrelationships and physiological roles are not clear.

In this study, we have identified and partially purified two different hydrogenase isoenzymes of *E. coli* in order to explore their relationships and physiological roles.

Materials and Methods

Materials

Sodium deoxycholate, sodium dodesylsulfate, methyl viologen, acrylamide and molecular weight marker proteins were purchased from Sigma; DE-52 from Whatman; hydroxyapatite from Bio-Rad; Sephadex G-200 from Pharmacia Fine Chemicals. And Triton X-100 was obtained from Merck.

Determination of Hydrogenase Activity and Protein Concentration

The hydrogenase activity was determined by the method of Choi and Yang. (1990) And the protein concentration was estimated by the dye-binding method. (Spector, 1978)

Enzyme Purification

E.coli HB101 cell (230g), which was grown aerobically in LB medium (1% NaCl, 1% Bactotrypton, 0.5% yeast-extract), was suspended in 400 m*l* of 50 mM Tris-Cl, pH 8.0, 5 mM benzamidine, 0.5 mM phenyl methyl sulfonyl fluoride, 2.5 mg /m*l* lysozyme and 0.1% 2-mercaptoethanol. The suspension was incubated at 37℃ for 1 hr and sonicated. After adding 13.5g of sodium deoxycholate, the solution was stirred at room temperature under hydrogen gas atmosphere and centrifuged at 10000rpm with Sorvall GSA rotor for 20 min. The supernatant were collected and 10-70% fraction of ammonium sulfate fractionation was dissolved in 250 m*l* of buffer A. (Buffer A: 50mM Tris-Cl, pH 7.5, 0.1% Triton X-100, 0.1% 2-mercaptoethanol, 5 mM benzamidine) The solution was dialyzed against buffer A, and stored at -20℃.

The crude extract was applied to a DE-52 column (3.5×18cm) which was equilibrated with buffer A. It was washed with 400ml of buffer A and eluted with 1 liter of 0-0.7M NaCl gradient at a flow rate of 13 m*l*6min. The resulting two activity peaks (hydrogenase M and L) were concentrated separately with Amicon PM10 membrane. Each isoenzymes were loaded again to a hydroxyapatite column (2.5×10cm) preequilibrated with buffer A. It was eluted with a linear gradient of 200 m*l* 0-0.7M potassium phosphate, pH 7.0 in buffer A at a flow rate of 0.59 m*l*/min. Those isoenzymes eluted from hydroxyapatite column were concentrated and further purified with Sephadex G-200 column. They were applied to a Sephadex G-200 column (1.77×35cm) and eluted with buffer A containing 0.1 M NaCl. The flow rate was 0.5 m*l*/3min.

Identification of Active Polypeptides by Two-Dimensional Electrophoresis

The polypeptides concerning hydrogenase activity were analyzed by two- dimensional polyacrylamide gel electrophoresis. The protein samples were electrophresed on a non-dissociating high-pH discontinuous polyacrylamide gel as a first dimension according to the method of Hammes. (1981) The gel was placed in a air-tight vessel containing 50 mM methyl viologen, 50 mM Tris-Cl, pH 7.5. It was flushed with hydrogen gas for 5 min and after adding sodium hydrosulfite solution until blue colour just appeared, it was incubated at 37℃. The hydrogenase activity was appeared as blue bands and they were permanently stained to red colour by the injection of 1 m*l* of 20 mM 2',3',5'-triphenyl tetrazolium chloride. The activity-stained gel was sliced and immersed in 0.0625M Tris-Cl, pH 7.0, 2% sodium dodesylsulfate, 5% 2-mercaptoethanol, 10% glycerol for 1 hr. They were electrophoresed on a SDS-polyacrylamide gel as a second dimension by the method of Hammes. (1981)

Results and Discussion

The elution profile of DE-52 column chromatography is shown in Fig. 1. Two major activity peaks appeared; the one in the first brownish protein peak and and the other between two protein peaks. They were named as hydrogenase M and L respectively. Hydrogenase M preparation was brownish solution and easily denatured during storage, whereas L was colorless. The elution proiles of hydroxyapatite and Sephadex G-200 column chromatography of isoenzyme M and L are shown in Fig. 2-5. The

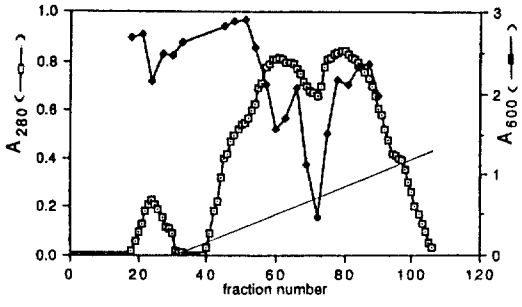

Fig. 1. *Elution profile of hydrogenase isoenzymes from DE-52 column. The prepared crude extract was applied to a DE-52 column (3.5×18 cm) preequilibrated with buffer A (described in Materials and Methods) and eluted with 0-0.7 M NaCl gradient. Each fraction contained 13 ml. The fractions 59-64 (for isoenzyme M) and 68-76 (for L) were collected and concentrated to 10 mls.*

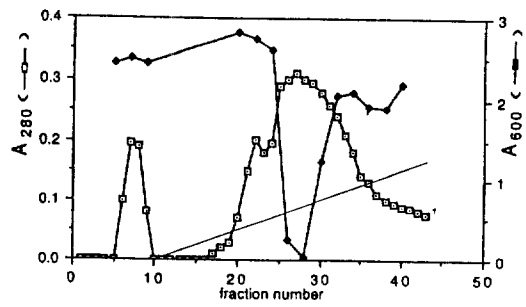

Fig. 2. *Elution profile of isoenzyme A from hydroxyapatite column. The isoenzyme M from DE-52 column, was applied to a hydroxyapatite column (2.5×10 cm) which was equilibrated with buffer A. It was eluted with 0-0.7M potassium phosphate gradient. The fractions 25-30 (35.4 ml) were collected and concentrated to 6 ml.*

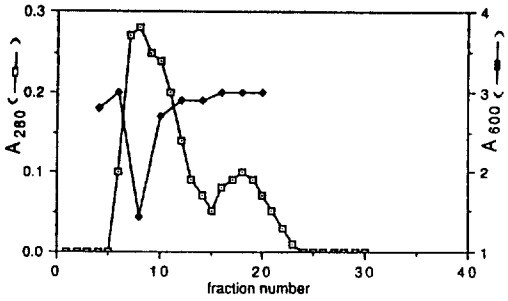

Fig. 3. *Elution profile of isoenzyme M from Sephadex G-200 column. The isoenzyme M from hydroxyapatite column, was applied to a Sephadex G-200 column (1.77×35 cm) which was equilibrated with buffer A and eluted with buffer A containing 0.1 M NaCl at a flow rate of 5 ml/fraction. 1 fraction/30 min. The fractions 7-9 were collected and concentrated to 3 ml.*

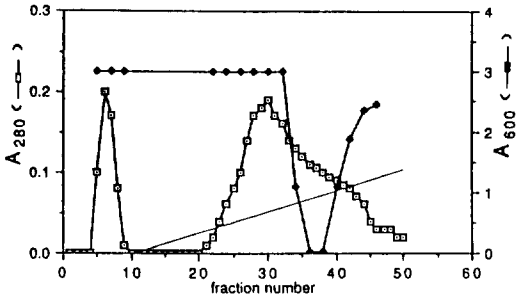

Fig. 4. *Elution profile of isoenzyme L from hydroxyapatite column. The isoenzyme L from DE-52 column, was purified using a hydroxyapatite column as described in Fig.2. The fractions 34-42 (53.1 ml) were collected and concentrated to 4.5 ml.*

isoenzyme L was eluted at higher phosphate concentration than M in hydroxyapatite column chromatography. The void volume of the Sephadex G-200 column was estimated to 32 ml

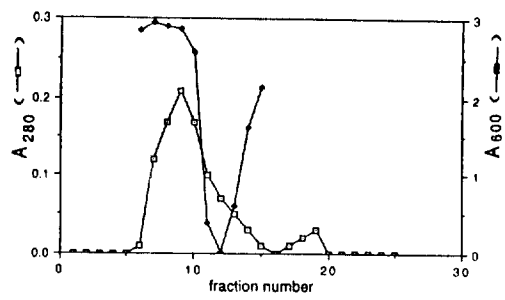

Fig. 5. *Elution profile of isoenzyme L from Sephadex G-200 column. The isoenzyme L from hydroxyapatite column was purified using Sephadex G-200 column as described in Fig. 3. The fractions 12-14 (15 ml) were concentrated to 1 ml.*

with Blue dextran 2000 and partition coefficients (Kav) of M and L were 0.15 and 0.52 respectively. The molecular masses of their native enzyme complex was roughly estimated to about 200 kDa and 50 kDa. The results are summarized in Table 1. The isoenzyme L had higher specific evolution activity than M. But, only M showed uptake activity after polyacrylamide gel electrophoresis. This indicates that L is the electrophoretically labile hydrogenase. The isoenzyme M was unstable and had lost its activity rapidly during purification by forming aggregates. Furthermore, it was easily solubilized when digested with trypsin or pancreatin, and the solubilized enzyme was stable. (Choi and Lee, 1991) These results indicate that the isoenzyme M is a membrane-bound protein and the instability may arise from its hydrophobic part. The isoenzyme L was only a minor protein.

The active polypeptides can be defined by increasing intensity during purification. Two groups of protein appeared in isoenzyme M preparation; the one in 70-75 kDa region and the other in 45-48 kDa (data not shown). On the other hand, two major protein bands, whose molecular masses are 31 and 27 kDa, were found in

Table 1. *Purification table for two hydrogenase isoenzymes*

Step	Isoenzyme	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg)	Yield (%)	Purification fold
Crude extract	total	740	700	0.95	100	1
DE-52	M	197	110	0.56	16	0.6
	L	118	97	0.82	14	0.6
HAP	M	129	46	0.36	6.6	0.64
	L	67.2	67	1.00	9.6	1.2
Sephadex	M	66	26	0.39	3.7	0.7
	L	2.2	21	9.5	3.0	11.6

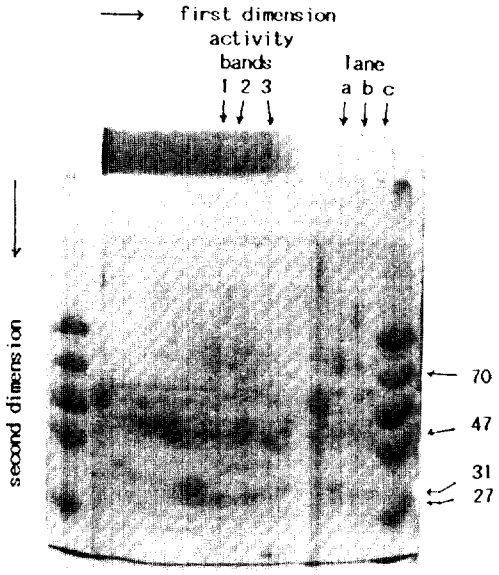

Fig. 6. *Identification of active polypeptides of detergent-solubilized crude extract by two-dimensional electrophoresis. The crude extract, prepared as described in Materials and Methods, was applied to a 7.5% non-dissociating high-pH polyacrylamide gel as a first dimension, electrophoresed at 200 V for 4 hours and stained against hydrogenase activity as described in Materials and M1 ethods. One whole lane was sliced and electrophoresed on a 7.5% discontinuous SDS-polyacrylamide gel as a second dimension. For comparison, the activity bands 1, 2 and 3 was sliced and electrophoresed in lanes a, b and c. It was stained with Coomasie brilliant blue. The marker proteins are same as in Fig. 6.*

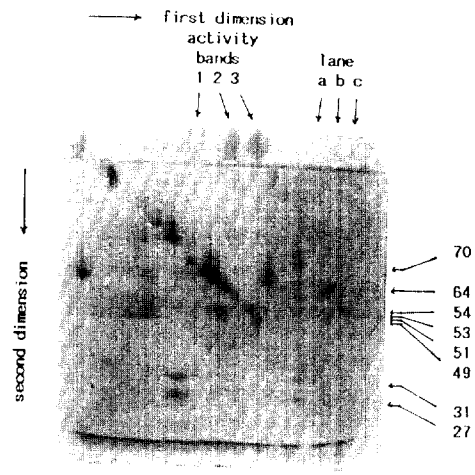

Fig. 7. *Identification of active polypeptides of pancreatin-solubilized crude extract by two-dimensional electrophoresis. The crude extract was treated with pancreatin (3.3 mg/ml) at 37 C for 1 hour and analyzed as in Fig. 7.*

isoenzyme L preparation (data not shown). The polypeptides were identified by the two-dimensional acrylamide gel electrophoresis. (Fig. 6) Three activity bands appeared after first dimensional electrophoresis. (indicated by arrows and numbers 1, 2 and 3) The band 3 was most thick. It was again electrophoresed on a SDS-polyacrylamide gel. The 70-kDa polypeptide is shown in band 1, 2 and weakly in 3, the 47-kDa polypeptide in all three activity bands and the 31- and 27-kDa polypeptides only in band 1. Another result was obtained with pancreatin-treated crude extract. (Fig. 7) Three activity bands appeared in the first dimension gel and the band 1 was most thick. They were analyzed with a second dimension gel by the same method as above. The band 1 contained 75-, 31- and 27-kDa polypeptides, band 2 contained 64-, 54-, 53-, 51- and 49-kDa and band 3 contained only 54-kDa polypeptide.

Three hydrogenase isoenzymes were identified by Sawers *et al.* (1985) Hydrogenase 2 activity was enhanced by fumarate and correlated with cellular H2-uptake activity. This implies the possibility that hydrogenase 2 is linked to fumarate reductase. In contrast, hydrogenase 1 activity was negligible without exogeneous formate and it was much more reduced in a pfl mutant, which is unable to synthesis formate. But, as a constituent of the formate hydrogenylase complex, third hydrogenase was postulated, because hydrogenase 1 was not essential for formate hydrogenylase activity. The isoenzyme 1 was purified after solubilization with sodium deoxycholate and Triton X-100) and was consisted of two polypeptides of Mr 64000 and 35000. (Sawers and Boxer, 1986) This result was confirmed by cloning and sequencing of the gene coding isoenzyme 1. (Menon et al. 1990) On the other hand, the isoenzyme 2 was solubilized with tryptic digestion and purified to two polypeptides of Mr 61000 and 30000. (Ballantine and Boxer, 1986) It was also reported that the membrane-bound hydrogenase of aerobically grown *E. coli* cells was solubilized by treatment with deoxycholate and pancreatin and was purified to a single polypeptide of Mr 56000. (Adams and Hall, 1979) Although the isoenzyme M agrees with neither 1 nor 2 which was purified by Boxer *et al.*, it may be a intact form of isoenzyme 2. It seems that 70-kDa polypeptide reduces to 61-kDa by digestion with trypsin and to 64- or 54-kDa with pancreatin. The 47-kDa polypeptide may not be a essential part

of hydrogenase, for it was removed by digestion with pancreatin. (Fig. 7).

An electrophoretically labile hydrogenase was partially characterized by Stoker *et al.* (1988). The molecular mass of this enzyme was estimated to about 300kDa by gel filtration. They also isolated a *E. coli* K-12 mutant which contained only 20% of the total hydrogenase activity, but showed normal activity banding pattern after polyacrylamide gel electrophoresis. In this mutant, H_2-uptake pathway was blocked, whereas H_2-production by the formate hydrogenylase was maintained. It was postulated, therefore, this electrophoretically labile hydrogenase was responsible for fumarate reductase activity. In contrast to this, another mutant was isolated, which lacked the labile hydrogenase activity (the third hydrogenase) and this characteristic was accompanied by a defect in formate hydrogenylase function. (Birkmann *et al.*, 1987) It is possible, then, that there are two labile hydrogenases concerning two metabolic pathways and our hydrogenase L is the third hydrogenase (Sawers *et al.*, 1985) because of its low molecular mass (50 kDa) and high H_2-evolution activity.

적 요

Detergent 들을 이용하여 대장균의 막에 결합된 수소발생효소를 부분정제하였다. 대장균 추출물을 sodium deoxycholate 로 처리한 후 Triton X-100 을 포함한 완충액에 대해 dialysis 하였다. DEAE-cellulose, hydroxyapatite, 그리고 Sephadex G-200 column chromatography 를 이용하여 정제한 결과 두 종류의 서로 다른 수소발생효소가 얻어졌다. 하나는 매우 불안정하였고 주 단백질로서 70- 과 47-kDa polypeptide 를 포함하고 있었으며, 또 하나는 수소발생활성이 높은 효소로서 31- 과 27-kDa 의 주 단백질을 포함하고 있었다. 이러한 polypeptide 들은 2차원 전기영동 분석법에 의해서 관찰되어졌다.

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