An Application of Isoelectric Focusing in the Purification of Thiamine-binding Protein of Escherichia coli

Takahiro NISHIMUNE and
Ryoji HAYASHI
Department of Microbiology, Yamaguchi University School of Medicine, Ube
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An amphoteric substance of relatively low molecular weight (approx. molecular weight 1,000) has become available recenty (L. K. B., Sweden), and the isoelectric points of those ampholite cover the pI range of 3-10. Purification of proteins or determination of pI of proteins using the ampholite as a carrier have already found numerous applications in the study of proteins from various sources[1)2)].

In a previous report we have proposed thiamine-binding protein as a possible factor in the uptake system of thiamine into E. coli cell[3)], but the content of thiamine-binding protein in bacterial cell was very small when compared to amino acid binding proteins or sugar binding proteins[7)], and thiamine-binding protein was always accompanied by a large amount of contaminating protein of pI extremely close to that of the binding protein.

A carrier ampholite covering the pH range of 0.35 was prepared and tested as a tool of purification of thiamine-binding protein and the result was described in this report.

MATERIALS AND METHODS

Carrier ampholite

The carrier ampholite (Ampholine®, 40% solution) was purchased from L. K. B. (Sweden), which covered the pH range of 5.5–6.2. The carrier ampholite was used as it was or after further fractionation to the pH range of 5.88–6.23 by the method of isoelectric focusing without protein solution according to the general directions of the manufacturer.

Isoelectric focusing equipment

The 440 ml electrofocusing column of L. K. B., which had been designated to facilitate the emptying with a minimum of disturbance, was used in combination with a voltage stabilizer and a fraction collector together with a constant temperature circulator (Toyo, Coolnit CL-15) in a cold room (4°C). pH of eluate fraction was measured by Hitachi-Horiba F-5 pH meter.

Thiamine-binding protein

Partially purified protein preparations of the specific activity of 0.610 nmole/mg (DEAE Sephadex A-50 column eluate) or 3.96 nmole/mg (BioGel CM-30 column eluate) were used.

Binding activity assay

Thiamine-binding activity was assayed by a modified equilibrium dialysis meth od[4]. In the case of polyacrylamide disc electrophoretic analysis the activity was detected, after slicing the gel into 3.3mm length portions, in the gel extract which was obtained by immersing the sliced gel in 1.0ml of 0.1M potassium phosphate buffer of pH 7.0 containing 0.5 mM β-mercaptoethanol overnight at 4°C.

Polyacrylamide disc electrophoresis

The standard method of Ornstein, Davis[5] was used. The proteins were stained by amido black 10B and destained electrically. The densitogram was obtained using a scanning light of the wave length of 600 nm.

RESULTS

Isoelectric fractionation in the carrier ampholite of pH 5.5–6.2

A protein solution (protein concentration : 1.66 mg/ml, total volume : 145 ml, specific activity : 0.610 nmole/mg) was fractionated according to the general directions of the manufacturer. Ethylene diamine and H_3PO_4 were used as light and dense electrode solutions, respectively, as follows ;

1. dense electrode solution : H_3PO_4 (85% or more) 0.8 ml, sucrose 48.0 g, H_2O 56.0 ml.
2. light electrode solution : Ethylene diamine 0.8 ml, H_2O 40.0 ml.
3. dense sucrose solution : Carrier ampholite (40%, pH 5.5–6.2, final concentration 0.7%) 5.25 ml, sucrose 100.0 g, H_2O 145 ml.
4. light sample solution : Carrier ampholite 1.75ml, sample solution 145ml, H_2O 69 ml.

1. was introduced at first into the bottom of the column followed by the mixed solution of 2. and 3. 2. and 3. were mixed to form a linear sucrose density gradient using a gradient mixer (L. K. B.) and a peristaltic pump. 4. was overlayered at the top of the column.

Electric current was supplied to the light and dense electrode solution as follows ; start : 500 V, 4.5 mA, at the end of the run (after 96 hours) : 810 V, 1.7 mA, the current was adjusted to a higher value from time to time.

After the electric current had turned off the column was emptied and fractionated to 4g fractions. pH, OD_{280nm} and thiamine-binding activity of each fraction were measured and recorded in Figure 1. As shown in the figure binding activity

was recovered in these fractions which show the pH value of 6.0 ± 0.05 and the isoelectric point of thiamine-binding protein was determined as pH 6.0.

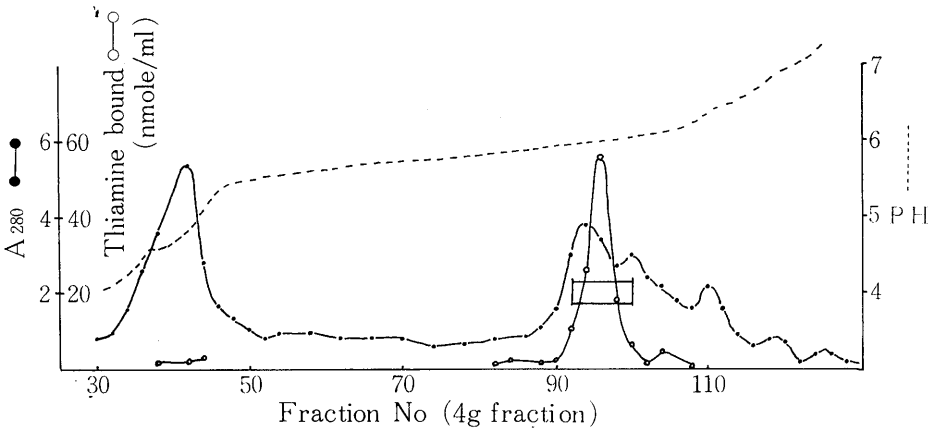


Fig. 1. Isoelectric fractionation of partially purified thiamine-binding protein in a carrier ampholite of pH 5.5–6.2.
Experimental details are described in the text. Fractions combined for recovery determination were indicated in the figure.

The result of isoelectric fractionation as a method of purification of thiamine-binding protein was summarized in Table I and the protein solution before and after the isoelectric fractionation were analyzed by polyacrylamide disc electrophoresis. The results were shown in Figure 2 as a densitogram. From the result it seemed that isoelectric fractionation could be one of the purification procedures of thiamine-binding protein, though it could not show an absolute preponderance over conventional methods.

Table I. Isoelectric fractionation of partially purified thiamine-binding protein by 0.7 pH range carrier ampholite

Sample	Protein (mg)	Specific activity (nmole/mg)	Recovery (%)	Purification (×)
Before fractionation	240	0.610	100	1
After fractionation	84.7	1.18	69	1.94

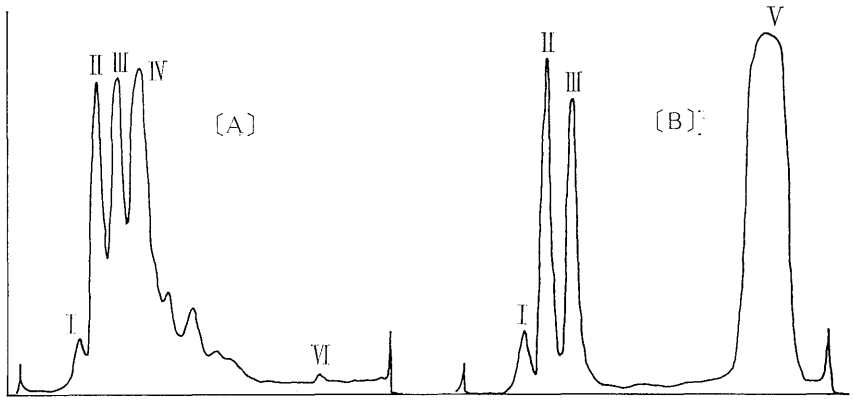


Fig. 2. Densitogram of the results of polyacrylamide disc electrophoresis.
[A] : The sample of partially purified thiamine-binding protein (specific acivity 0.610 nmole/mg) was analyzed before isoelectric fractionation.
[B] : The same sample as [A] was analyzed after isoelectric fractionation. Peak IV and minor peaks of [A] had been eliminated but peak II and III are still present. Peak V can be removed by dialysis.
I : thiamine-binding protein, II-IV : contaminating proteins, V : carrier ampholite, VI : bromphenol blue.

Isoelectric fractionation in the ampholite of a narrower pH range

Preparation of the carrier ampholite of a narrower pH range (pH 5.88–6.23) : The same experiment as described above was carried out with double the amount of carrier ampholite (3. : carrier ampholite 10.5 ml, sucrose 100 g, H_2O 139 ml, 4. : carrier ampholite 3.5 ml, H_2O 211 ml) without adding protein solution. The result of fractionation was shown in Figure 3. The fractions of pH 5.88–6.23 were combined and used in the experiment described below.

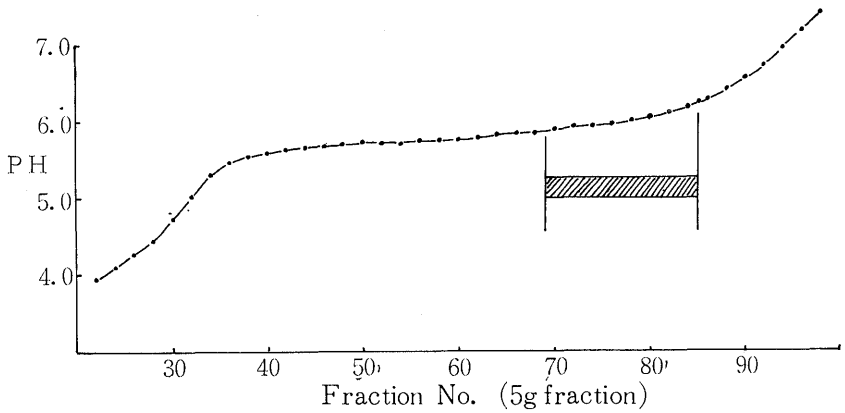


Fig. 3. Preparation of narrower pH range ampholite.
Experimental details are described in the text. The fractions indicated in the figure were combined and used in the experiment shown in Fig. 4.

Preparation of a narrower pH range column: The four component solutions were modified as follows;

1. H_3PO_4 (85% or more) 0.80 ml, sucrose 50.0 g, H_2O 56.0 ml.
2. Ethylene diamine 0.20 ml, H_2O 40.0 ml.
3. Carrier ampholite (combined fractions obtained above) 55.5 ml, sucrose 100 g, carrier ampholite of pH 5.5–6.2 (40%) 0.6 ml, H_2O 94.5 ml.
4. Carrier ampholite (combined fractions obtained above) 18.5ml, carrier ampholite of pH 5.5–6.2 (40%) 0.2 ml, sample solution (protein 136 mg in 1 mM potassium phosphate, pH 7.2, specific activity 3.96 nmole/mg) 141 ml, H_2O 55.5 ml.

Isoelectric focusing: The electric current of 500 V, 3.0 mA was applied at the start, and after 144 hours of occasional adjustment of voltage to a higher value, the current was 660 V, 0.43 mA. The voltage should be raised very slowly to avoid the formation of eddy zones of local overheating. After 144 hours of power supply the column was emptied as the previous experiment and fractionated into 4 g fractions. The results were shown in Figure 4. As shown in the figure thiamine-binding activity was detected in two different group of fractions. The results were summarized in Table II.

The concentration of carrier ampholite in the above described experiment was estimated as approx. 0.45 % on the basis that ca. 1/3 volume (cf. Fig. 3) of the 1.4% carrier ampholite was used for the experiment.

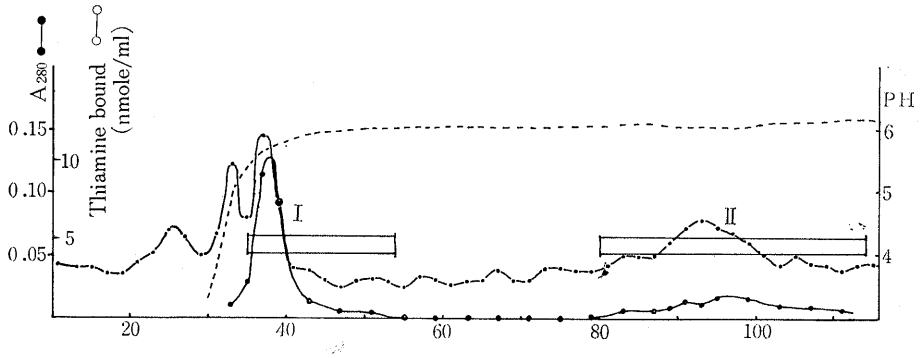


Fig. 4. Isoelectric fractionation of partially purified thiamine-binding protein in a carrier ampholite of pH 5.88–6.23.

Experimental details are described in the text. Thiamine-binding activity was detected in a main peak (I) and a minor, broad peak (II). The fractions combined for recovery determination were indicated in the figure.

Table II. Isoelectric fractionation of partially purified thiamine-binding protein by 0.35 pH range carrier ampholite

Sample	Protein (mg)	Specific activity (nmole/mg)	Recovery (%)	Purification (×)
Before fractionation	136	3.96	100	1
After fractionation	31.0	8.84	51 (74)	2.23

The recovery was calculated by combining the main activity peak and the side activity peak

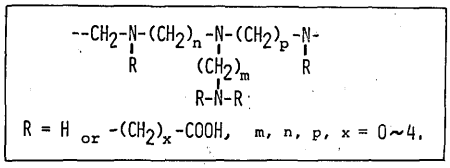
DISCUSSION

It is possible, theoretically, to prepare any carrier ampholite covering very small range of pH, and therefore to increase the resolving power of the method against protein mixture indefinitely. In practice, however, the concentration of carrier ampholite, which can be prepared from the 40 % carrier ampholite of 0.5 pH range (available commercially), become too small when narrower pH range than 0.5 is adopted.

In the experiment using a narrower pH range ampholite (Fig. 4) the binding activity was recovered in two peaks. Though in the majour peak the fractions carrying the binding activity were restricted enough, the second peak containing the activity was distributed amoung very broad range.

The carrier ampholite of too low concentration will not be able to keep a buffering capacity exceeding that of the proteins, which means that they can not determine the pH in the system and the pH of the proteins dominate. In such a circumstance each protein component will not be concentrated as a shallow zone and spreaded among broad fractions.

Carrier ampholite is reported to be composed mainly of a number of different aliphatic polyamino-polycarboxylic acids and have been represented by the next formula[6].



Those carrier ampholite was stained by amido black 10 B (cf. Fig. 2 [B]) and absorbed the UV light of the wave length of 280 nm (cf. Fig. 1). Thus the sample for polyacrylamide disc electrophoresis should be dialyzed to remove those ampholite before analysis.

Isoelectric fractionation was not effective dramatically as a purification procedure of thiamine-binding protein. The main reason of this seems to be the existence of a large amount of protein of very close isoelectric point to that of thiamine-binding protein (within 0.02–0.04 pH, Fig. 2 [B]–II, III). This contaminating protein has been removed with great difficulties[7] by many successive conventional purification procedures.

The degree of purification was not so improved by the use of a narrower pH range (0.35) carrier ampholite, in the multiple of specific activity, when compared to the result obtained with the use of 0.7 pH range carrier ampholite (cf. Table I and Table II, 2.23 : 1.94). But the absolute specific activity of the sample used in the former experiment was ca. 6 times higher than that in the latter experiment, and the specific activity of the product obtained in the former experiment was ca. 8 times higher than that in the latter experiment (8.84 : 1.18).

SUMMARY

A partially purified thiamine-binding protein preparation was fractionated by isoelectric fractionation and following results were obtained.

1. The isoelectric fractionation could be used as a method of purification of the binding protein with nearly the same efficiency and recovery as those of conventional purification methods, but it took at least 3–5 days for an experiment.
2. The carrier ampholite covering the pH range of 0.7 (pH 5.5–6.2, 40% concentration) was used in a final concentration of 0.7% with a 440 ml column and was found adequate for the fractionation of 240 mg of proteins, which had been liberated from E. coli cells by osmotic shock.
3. The carrier ampholite of the pH range of 0.35 (pH 5.88–6.23) was prepared and used for the binding protein purification. The final concentration of the ampholite was approx. 0.45 % and thiamine-binding activity was recovered in two separate groups of fractions. The recovery in the main activity peak was 51 % with 2.2 times purification in the specific activity and could be used as a purification procedure.
4. The carrier ampholite of narrower pH range (0.35 pH) gave a fractionation product of the specific activity of 8.84 nmole/mg from the sample of 3.96 nmole/mg. The carrier ampholite of 0.7 pH range gave a fractionation product of the specific activity of 1.18 nmole/mg from the sample of 0.61 nmole/mg. Those values indicated that the resolving power of the narrower pH range ampholite was superior to the 0.7 pH range ampholite appreciably.

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