Studies on Acetoacetate Decarboxylase by the Polyacrylamide Gel Disc Electrophoresis

—Activity Staining Method of Acetoacetate Decarboxylase—

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Acetoacetate decarboxylase (EC 4. 1. 1. 4) catalyzes a reaction which cleaves acetoacetate to acetone and carbon dioxide. The enzyme was first crystallized by Hamilton and Westheimer[1]. The reaction kinetics, active site structure and physicochemical properties of the enzyme have been studied by various investigators[2),3),4),5)]. In the course of the studies of substrate specificity, we purified the enzyme by modifing the method of Westheimer et al[6]. The purification steps were followed by polyacrylamide gel discelectrophoresis. In order to demonstrate the enzyme activity in the electrophoresis gel, we developed a specific stain method using pH indicators. The most effective pH indicator was BTB. Using BTB as indicator, 0.02 unit of the enzyme activity could be detected as a sharp band.

MATERIALS AND METHODS

I Materials

BTB, CR, PR, NP, MR and PP were purchased from Katayama Chemical Co., Yeast extract from Oriental Yeast Co., polypeptone from Daigo Eiyo Co., TGC medium from Eiken Chemical Co., DEAE-cellulose (0.74 meq/g) from Serva, ammonium sulfate and Amide-Black 10B from Merck, silicone emulsion from Toray-Daucorning. Sucrose was recrystallized in our laboratory. Acetoacetate was obtained by hydrolyzing ethylacetoacetate. Other chemicals were all of analytical grade prepared in Japan. The microorganism *Clostridium acetobutylicum* (ATCC 862) was purchased from American Type Culture Collection.

II Polyacrylamide gel disc electrophoresis

The polyacrylamide gel disc electrophoresis was performed using

Abbreviations: BTB=bromothymol blue, CR=cresol red, PR=phenol red, NP=p-nitrophenol, MR=methyl red, PP=phenolphthalein, TGC medium=thioglycolate culture medium, DEAE-cellulose=diethylaminoethyl cellulose, BPB=bromophenol blue.

modified method of Ornstein[7] and Davies[8]. A glass tube of 85mm in length and 5mm in diameter was used. 5cm of the small pore gel of 7.5% polyacrylamide, pH 8.9 and 1cm of large pore gel of 2.0% polyacrylamide, pH 6.7 were prepared. Sample solution was prepared by adding the diluted enzyme solution to three fourth volume of 60mM thioglycolate solution and three fouth volume of 50% sucrose (w/w) solution. 0.05–2 units of acetoacetate decarboxylase was applied on the top of the spacer gel under the running buffer solution.

Electophoresis was carried out using a Tris-HCl buffer of pH 8.3 at 200V 2mA per tube at room temperature. When the tracking BPB band migrated 4cm into the small pore gel, the electrophoresis was stopped and the one group of gels were stained with 1% Amide-Black 10B in 7% acetic acid while the others were stained by the activity staining solution as follows:

III Activity staining

After electrophoresis, the gel was incubated in the following dye solution for 3hr with gentle stirring in a cold room.

Dye buffer solution	0.05M acetate buffer pH 5.0	135ml
	saturated dye solution	15ml

Incubation in the dye buffer solution, because of the alkalinity of the electrophoresis gel (pH 9.5) the gel was at first stained to the alkaline color. The gel was neutralized by large volume of dye "buffer" solution. Then it gradually became an acid color, from the outer part to center. When the whole gel changed to this acid color, it was incubated into the substrate solution of the following composition. The activity band of alkaline color appeared immediately.

Substrate solution	2M	acetic acid	0.3ml
	1M	sodium acetoacetate	9.7ml
		(pH adjust to around 5.0)	

IV Measurement of acetoacetate decarboxylase activity

Enzyme activity was assayed by measurement of CO_2 evolved by Warburg manometer[6]. Into the main chanber were added 0.3ml of 2M acetate buffer of pH 5.0, 0.5ml of 0.5M sodium acetoacetate and 2.1ml of H_2O and into the side arm was added 0.1ml of enzyme solution (0.01–0.2 unit). After the temperature of the manometer was equiliblated at 37°C, the solution in the side arm was mixed. Controls without enzyme were used. One enzyme unit was defined as the amount which liberated 14.5μ mole of CO_2 per minute at 37°C under the experimented conditions[6]. Specific activity was defined by the enzyme activity per mg protein which were measured by the method of Lowry et al..

V Culture of Clostridium acetobutylicum

The culture procedure consisted of four steps. The state of fermentation at each step of inoculation was particularly important. To obtain highly active samples, innoculation had to be done when the culture reached the logarithmic phase, or the beginning of the next fermentation would have been retarded and the specific activity of the sample would have been low.

A) Stock culture

Ten ml of the stock culture medium composed of 1g of TGC medium, 0.5g of glucose, 1g of $CaCO_3$, 1g of agar, 20ml of potato extract* and 80ml of water were autoclaved at 120°C 30min. Since *Clostridium* was anaerobe, innoculation was carried out as soon as possible after autoclaving. Thus, the tubes that were still hot were cooled to room temperature by dipping them into an ice bath. The inoculated tubes were stored at 37°C. The gas formation was observed within 20hr. The culture could be stocked for three weeks at room temperature. It was necessary to inoculate into a new medium every 2 or 3 week for the maintenance of the strain.

*potato extract

Potatoes, chopped to pieces, were boiled 30 min with addition of 2 times of water (w/w). After grinding the pieces, the resultant paste was boiled again 30 min and filtered through a gause. The filtrate was very suitable for the growth of this bacteria.

B) First culture

First culture was made from the stock culture which was at a highly active stage of fermentation. Test tubes containing 10ml of the first culture medium composed of 1 g of TGC medium, 0.5g of glucose, 0.1g of agar, 0.1g of $CaCO_3$, 20ml of potato extract and 80ml of water were autoclaved, cooled as described above, and inoculated by the stock culture. Vigorous gas evolution occured within 24hr. This culture solution was transferred to the second culture.

The compositions of the growth media for the second, third and fourth culture were as follows;

Solution A	
Polypeptone	720 g
Yeast extract	36 g
L-asparagine	21.6 g
Water	2.5 l
Solution B	
Potassium phosphate monobasic	36 g
Salt solution**	360 ml
$FeSO_4$	720 mg

Ascorbic acid		720 mg
Silicone emulsion		2 ml
Saturated methylene blue solution		2 ml
Water		12 l
Solution C		
Sucrose		720 g
Water		2.5 l
** Salt solution	$MgSO_4 \cdot 7H_2O$	40 g
	$MnSO_4 \cdot H_2O$	1.24 g
	NaCl	2 g
	add H_2O to 1,000 ml	

Solution A, B and C were hitherto prepared separately but they were mixed before autoclaving. The mixed medium was stocked with small amounts of toluene within a week. After autoclaving for 30–120min at 120°C, the medium was cooled as soon as possible to room temperature to prevent the mixing of oxygen. It was stocked overnight before being used.

C) Second culture

Four hundred ml of the growth medium in 500ml Erlenmeyer flask were autoclaved at 120°C for 30min and cooled to room temperature, as described above. When this manipulation was inadequate, the methylene blue in the medium changed to green. The greenish medium must be re-autoclaved to drive out the oxygen. But, the medium which was autoclaved more than 2 times was not suitable for the culture. When the first culture solution fermenting actively was transferred into second culture, vigorous fermentation was seen within 12hr.

D) Third culture

Five l. of the growth medium were autoclaved at 120°C for 1hr. Into the cooled medium was transferred the second culture solution. After 8–10hr, the fermentation was observed; each 500ml of this culture solution was transferred to the fourth culture medium.

E) Fourth culture

Ten l. polyethylene bottle was used for the manipulation of the fourth culture. Ten bottles containing each 10 l. of the growth medium were cooled to around 37°C, the caps were firmly closed. It usually required approximately 40 min. During this time, a considerable amount of air oxygen probably dissolved into the medium, but the bacterial contamination was negligible.

Some sodium hydrosulfite was added to the medium to lower the oxygen content of the medium (the amount was checked by the color change of the medium from dark brown to light brown; 0.3–0.5 g/10 l. medium was needed). Then, each 500ml of active cell solution from third

culture was transferred to the fourth culture medium. In the mixtures incubated at 37°C, vigorous fermentation occured within 13hr and ended within 50hr, when the gas evolution ceased and precipitation of the bacteria was seen in the bottle.

VI Preparation of acetone powder

The fourth culture solution was centrifuged at 10,000 rpm 4°C, and the cells obtained were washed once with 400ml of cold water. About 400g of cells were obtained. The cells were suspended in the same weight of water and 10 times its volume of chilled acetone was added with vigorous stirring. After 5 minutes, the cells were filtrated through Buchner funnel and the precipitate was washed with cold acetone and then with cold ether. The dehydrated bacterial mass was ground in a mortar and dried in vacuo overnight in a refrigerator. The granular substance was ground to a fine powder and dried for 6hr in vacuo. The powder obtained had 30% of the wet weight of the cells and maintained its activity for about 6 months in a deep freezer at −20°C, without appreciable loss of activity.

RESULTS AND DISCUSSION

I Activity staining

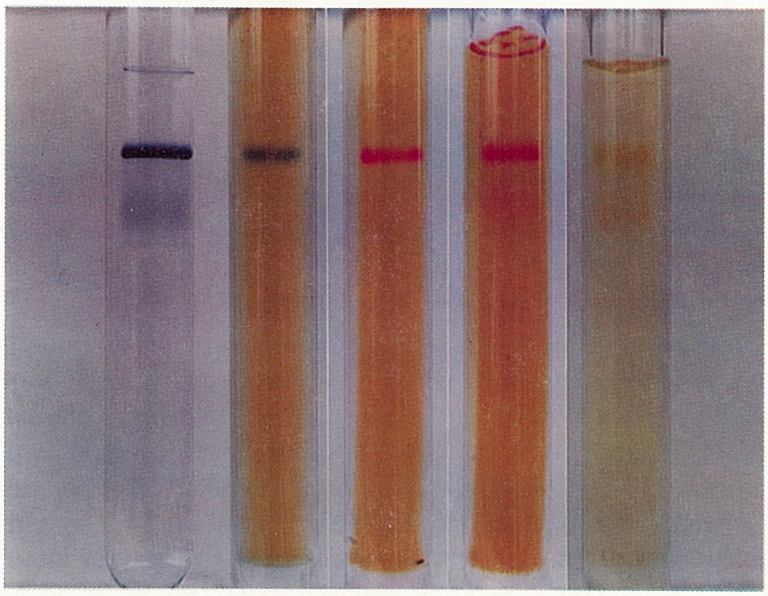
When the gel is incubated in a substrate solution, acetoacetate is decomposed to CO_2 and acetone on the enzyme band, making the solution in the region more alkaline, since CO_2 (pK=6.35) dissociates far less than acetoacetic acid (pK=3.83). Thus, the band of acetoacetate decarboxylase in the gel will be visualized by a pH indicator.

Several pH indicators which have the range of color change between pH 5.0 to 8.5 were investigated for the gel. As will be shown later, the color band remained in all the fractions obtained by various purification steps of the enzyme, with its color intensity becomming stronger, as the enzyme became more purified, and at least the color band corresponded to the only protein band of the crystalline preparation of the enzyme. Hence it is concluded that the color band represented the enzyme band and that the staining method was suitable for the visualization of acetoacetate decarboxylase. In table 1, the color of the decarboxylase band and that of the background are shown. PP and MR showed no detectable color band, although they have the range of color change between pH 8.3–10.0 and pH 4.2–6.2, respectively. The reasons may be as follows: MR changes from red to yellow, between pH 4.2 to 6.2. Hence the background is already faintly yellowish, which may obscure the change of the band color. Furthermore, since MR is hardly soluble in buffer solution at pH 5, the solution of

sufficient concentration of MR for the activity staining could not be possible to prepare. On the other hand, PP changes its color in the range of pH 8.2 to 10.0. Thus the pH change which occure at the enzyme band would not reach the value needed for the color change.

Table 1. pH indicators and the color of the band

pH indicator	background	range	color of the band
BTB	yellow	pH 6.0– 7.6	blue
CR	yellow	7.2– 8.8	red
PR	yellow	6.8– 8.4	red
NP	colorless	5.0– 7.0	yellow
MR	colorless	4.2– 6.2	—
PP	colorless	8.3–10.0	—



A B C D E

Fig. 1. Polyacrylamide gel disc electrophoresis of crude crystal preparation of Acetoacetate decarboxylase from Clostridium acetobutylicum. 0.4 unit (10 μg) of the enzyme was used for each gel. A: protein stain, B, C, D and E: activity staining using BTB(B), CR(C), PR(D) and NP(E), respectively.

BTB, CR and PR were all suitable for the detection of acetoacetate decarboxylase as seen in Fig. 1. Among them, the most clear band was obtained by BTB (Fig. 1-B) which could detect 0.02 unit of the enzyme (0.5 μg). CR (Fig. 1-C) was about four times weaker than BTB in sensitivity. We may expect, that the best indicators for the detection of acrtoacetate decarboxylase may be those which have the range of color change between pH 5-7, and easily decernible color change, e. g. from yellow to blue such as BTB.

When the gel was suspended in the substrate solution, the color band appeared immediately. The intensity of the color of the band increased during 30 min but the color contrast became weaker, probably because of the rapid diffusion of pH gradient. The color contrast was sharpest in the 5-7 min incubation period. Hence the gel was photographed at that time. Fig. 1 shows the active bands obtained by this activity staining.

The molecular weight of the enzyme was reported to be about 300,000[9] and hence the diffusion of this enzyme in 7.5% polyacrylamide gel appeared to be strongly limited. After a 3hr incubation at pH 5.0, the protein band was sharp as in the gel that was not incubated. If a shorter incubation time is preferable, a more concentrated buffer solution could be used. The gel was incubated for one minute in a 1M acetate buffer pH 5.0 saturated with the pH indicator, incubated in a water for 4 min and then incubated in the substrate solution. The color band appeared immediately from the outer side of the gel, in ring form. However, this rapid method is less sensitive and it can be used only for a large amount of enzyme (above 0.2 unit).

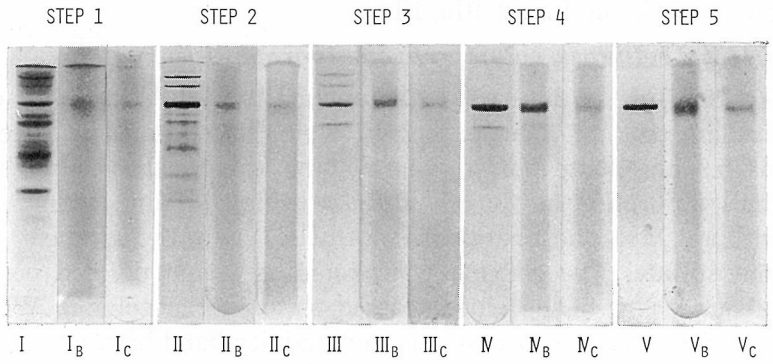


Fig. 2. Polyacrylamide gel disc electrophoresis at vaious steps of crystallization of Acetoacetate decarboxylase from Clostridium acetobutylicum. Three electrophoretic patterns are demonstrated in each purification steps. The left (I, ····· V) is protein stain, the middle (I_B, ····· V_B) is the color band by BTB and the right (I_C, ····· V_C) is by CR.

II Crystallization steps and the detection ofthe active band

Step 1 Preparation of the crude extract

One hundred g of acetone powder were extracted with 1500 ml of 0.05 M phosphate buffer of pH 5.9 on a shaker for 2hr at 37°C. The suspension was centrifuged at 4,000 rpm for 30 min. About 1200ml of the crude extract were obtained. As seen in Fig. 2, about 20 protein bands (Fig. 2–I) were seen usually in the crude extract and an active band by BTB stain (Fig. 2–I_B) was visualized in accord with the 6th band from cathodic side of gel. The color of the band with CR (Fig. 2–Ic) was very weak. In Fig. 2, protein and activity stains of other steps of the purification were also demonstrated.

Step 2 Isoelectric precipitation

The pH of the crude extract was lowered to pH 3.8 with 2M acetic acid. After 30 min, the precipitate was collected by centrifugation at 6,000 rpm for 30 min and resuspended in a small amount of 0.05M phosphate buffer pH 5.9 and centrifuged at 10,000 rpm. Much protein failed to dissolve, and the extraction was repeated several times, even with 0.1M phosphate buffer, in order to dissolve the enzyme. In activity staining, CR presents a band which is comparatively clear (Fig. 2–IIc).

Step 3 Ammonium sulfate fractionation

About 400ml of combined supernate were made to 60% saturation by adding solid ammonium sulfate. The precipitate collected by centrifugation was dissolved in approximately 10ml of 0.05M phosphate buffer of pH 5.9 and dialyzed against the same buffer. The precipitate which appeared was removed by centrifugation. In this step, the each band is slightly enhanced in the activity staining (Fig. 2–IIIB, IIIc).

Step 4 DEAE-cellulose column chromatography

About 10ml of the supernate was applied to a DEAE-cellulose column (2.5cm in diameter and 40cm in length), which had been equilibrated with 0.05 M pH 5.9 phosphate buffer. The column was washed with the same buffer until absorption at 280 nm in the washing became negligible, and eluted with a 0.05 M phosphate buffer of pH 5.9 with the gradient of 0.1M ammonium sulfate. The enzyme was eluted as a well defined peak, and usually about 300ml of the eluent passed through the column. When the specific activity exceeded 30unit/mg, the activity band became very clear, especially in the case of BTB stain Fig. 2–IVB).

Step 5 Crystallization

About 150 ml of active fraction were collected and brought to 70% saturation with ammonium sulfate. The precipitate was collected by

centrifugation at 10,000 rpm. It was again dissolved in 1 to 2ml of 0.05 M phosphate buffer pH 5.9. Crystals began to precipitate within 30 minutes. Hexagonal crystals are seen in Fig. 3. The preparation appeared to be homogenous on the electrophoresis (Fig. 2–V). In activity staining (Fig. 2–VB, VC), the bands are very clear but rather obscure than step 4 because the photograph was taken 15min after colorization.

From 508g of acetone powder of Clostridium acetobutylicum, 17.7mg of crude crystals were obtained. Table II shows the steps of the purification. As shown in the Table, the yield of the enzyme on step 2 was only 30%, while that of Westheimer et al 70%[6]. The percentage (30%) of the

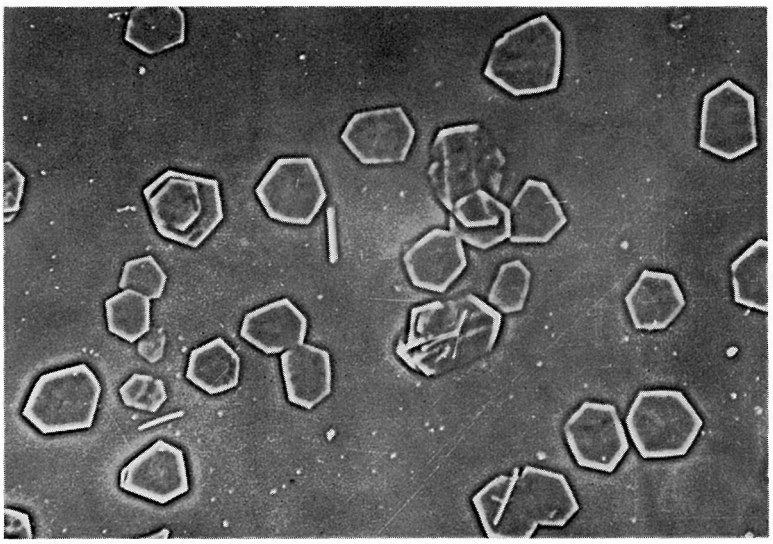


Fig. 3. Photomicrograph of crystals of acetoacetate decarboxylase ×400

Table 2. Purification steps of Acetoacetate decarboxylase

Step	Total protein	Total units	Specific activity	Yield
A: Crude extract	3919 mg	5140 U	1.34 U/mg	100%
B: Precipitation at pH 3.8	93.5	1560	16.7	29.9
C: 60% Saturation with $(NH_4)_2SO_4$	44.2	1021	23.1	19.9
D: DEAE-cellulose column chromatography	19.8	754	38.1	14.7
E: Crystallization	17.7	695	39.2	13.5
E: Recrystallization	10.1	399	39.5	7.8

yield did not improve through several purifications. In such cases, the crude extract had generally a specific activity of less than 0.5 unit/ml and most of the activity (98–99%) remained in the supernate. We, therefore tried to purify the enzyme from the supernate of step 2, but the improvement of the specific activity was only slight, and the yield of the crystal was 2% or less. When 30% of the total activity was transferred to the precipitate, the rest was inactivated, as there was no activity in the supernate. In any way, the method of isoelectric precipitation of the enzyme devised by Westheimer et al was not practical, especially for the initial materials of low specific activity. We found it to be more convenient to precipitate the protein from the crude extract with ammonium sulfate at 30–50% saturation. By this procedure, activity was recovered almost qauntitatively. The precipitation of the enzyme at the isoelectric point was effective, but probably in the case of an excellent culture. This can be deduced from the fact that the percentage of the precipitated activity from crude extract was roughly proportinal to the specific activity of acetone powder.

SUMMARY

The activity staining method for acetoacetate decarboxylase (EC4. 1. 1. 4) was developed by the use of acrylamide gel disc electrophoresis. The gel was immersed in a buffer solution of a pH indicator dye of pH 5.0. It was then incubated in the substrate solution. As the pH changes on the enzyme band due to the decomposition of acetoacetate to acetone and carbon dioxide, an alkaline colored band appeared in the acid colored background of the gel. pH indicator dyes tested were BTB, CR, PR, NP, MR and PP. Among them, BTB was most sensitive and MR and PP were ineffective.

The activity staining method described here was applied to the purification steps of the acetoacetate decarbozylase and was proven to be useful. Culture method of Clostridium acetobutylicum and purification of the enzyme from it were also discussed.

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