Purification of Aconitase

Hatanori OGATA and Shojiro NAKAMURA
Department of Biochemistry, Yamaguchi University School of Medicine, Ube, Japan
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Aconitate hydratase (EC 4. 2. 1. 3) was discovered by Martius and Knoop[1] to catalyze the following reactions:

$$\text{Citric acid} \underset{+\,H_2O}{\overset{-\,H_2O}{\rightleftharpoons}} \text{cis-Aconitic acid} \underset{-\,H_2O}{\overset{+\,H_2O}{\rightleftharpoons}} \text{iso-Citric acid}$$

The name "aconitase" was coined by Brausch[2] but the use of this name now is not recommended. Ochoa[3] concentrated the enzyme by ammonium sulfate precipitation from swine heart extract, but he did not attempt further purification. Buchanan and Anfinsen[4] attained a 23-fold purification of aconitase by low analysis showed that this preparation temperature ethanol and ammonium sulfate fractionations. Electrophoretic analysis showed that this preparation consisted of three components and the purity of the enzyme prepration was estimated to be 30%. Morrison[5] attained a 24-fold purification by low temperature ethanol and ammonium sulfate fractionation combined with heat fractionation. The purity of the final preparation was estimated to be about 78–80%. Henson and Cleland[6] attained a 20.5-fold purification of aconitase from beef liver by ammonium sulfate fractionation and carboxymethyl cellulose column chromatography. Disc electrophoretic analysis on polyacrylamide gel showed that the purified enzyme preparation consisted of four anionic components. From the relative amounts of them at different stages of purification, the major band which contained 60–70% of the total protein was regarded to be aconitase. Recently Villafranca and Mildvan[7] attained 76-fold purification of aconitase from swine heart by carboxymethyl cellulose chromatography, ammonium sulfate fractionation and Sephadex G 200 chromatography. Kennedy, Rauner and Gawron[8] also attempted the purification of aconitase from swine heart and attained 26.8-fold purification by ethanol fractionation, carboxymethyl cellulose chromatography, ammonium sulfate fractionation and isoelectric forcusing.

In succession to the studies of specificity of fumarase[9], we have attempted to at first purify aconitase. and then investigate the specificity and mechanism of the enzyme On the purification of aconitase, it was

found by disc electrophoresis, that swine heart extraction contained two different forms of aconitase and one was isolated and found to be apparently homogeneous.

MATERIALS AND METHODS

cis-Aconitic acid, Tris (tris (hydroxymethyl) aminomethane), NADP, nitroblue tetrazolium, phenazine methosulfate, systein, and iso-citrate dehydrogenase were purchased from Sigma Chemical Co. Other reagents were obtained from Katayama Chemical Co. Sephadex G-200, DEAE-Sephadex A-50, CM-Sephadex C-50, and Blue Dextran 2000 were purchased from Pharmacia. Hydroxy apatite was prepared using Levin's method[10]. Amido Black 10 B was purchased from Merck.

Estimation of enzyme activity: The citrate dehydrating activity was measured by Recker's photometric method[11]. The reaction mixture, consisting of 1 ml of 100 mM sodium citrate, 1 ml of 33.3 mM Tris-acetate buffer, pH 7.4, and 1 ml of an enzyme solution, was incubated in a cuvette of 1 cm light path at 25°C, and the absorbance was measured at 240 nm with a Hitachi recording spectrophotometer. One unit (U) of activity was defined as that which caused a change of absorbance of 0.01 per min.

Estimation of protein: The protein concentration of aconitase solution was determined by the measurement of the absorbance with a Hitachi spectrophotometer in a cuvette of 1 cm light path at 280 nm. One unit (d) of absorbance of an enzyme solution corresponded to 1.6 mg of protein per 1 ml according to the procedure of Buchanan and Anfinsen[4]. The specific activity was defined by the activity per unit absorbance of the enzyme solution.

Electrophoresis: 1) Disc electrophoresis of an enzyme preparation was carried out in glass tube of 5×80 mm, using the procedure of Ornstein and Davis[11]. The acrylamide concentration of separating gel was 7% and a sample solution with 50% sucrose and 60 mM thioglycolate was applied without sample gel on top of stacking gel. After electrophoresis, some disc gels were stained with Amido Black 10 B and destained electrophoretically. Some gels were stained by the aconitase activity as follows: The gels were incubated in a cold 50 mM Tris-HCl buffer solution (pH 7.8) containing 1 mM $FeSO_4$ for 10 min., washed with 50 mM Tris-HCl buffer solution (without $FeSO_4$), and incubated at 37°C in the dark in 10 ml of the aconitase staining solution containing 5.0 ml of 100 mM Tris-HCl buffer solution, 174 mg of cis-aconitate, 0.5 mg of iso-citrate dehydrogenase, 8 mg of NADP, 6 μ moles of $MnCl_2$, 4 mg of nitroblue tetrazolium, and 0.24 mg of phenazine methosulfate. When violet band appeared in the gel, the

reaction was stopped by throwing the gel into a 7% acetic acid solution.

2) For the enzymatic analysis of aconitase fraction in the gel, disc electrophoresis was carried out in a glass tube of 15×70 mm. After electrophoresis, the disc gel was inserted into a tube of acrylic plastic with a planger which could be driven by a screw, and the gel was cut by Inox No. 012 surgical stainless wire of 0.12 mm diameter, into 2 mm sections. Each section was dialyzed against a dialysis solution (4 mM citric acid and 14.5 mM Tris, adjusted to pH 7.4 with ammonia water-called afterwards, 4 mM C-T buffer solution, containing 10 mM cystein and 0.5 mM ferrous ammonium sulfate) in a cellophane tube containing 2 ml of the same buffer solution. After 15 hours the dialysis solution was changed to a solution of 4 mM C-T buffer solution, and the dialysis was continued for 5 hours. Aconitase activity of the solution in the tube was measured.

RESULTS

1. Preparation of a crude emzyme solution: Five swine hearts were freed of fat and connective tissues, minced, and homogenized for 10 min. in three times its weight of cold 4 mM C-T buffer solution and 1/10 of chloroform. The homogenate was centrifuged with a continuous centrifuge (Kubota Co.) at 9,000 r.p.m. in a cold room. The supernatant was fractionated with solid ammonium sulfate between 60–75% saturation as follows: The crude extract was mixed with 360 g/l of ammonium sulfate, stirred for 20 min., and centrifuged at 9,000 r.p.m. The precipitate was discarded. The supernatant was brought to 75% saturation with 105 g/l of ammonium sulfate, stirred for 30 min., and centrifuged. The precipitate obtained was suspended in a 75% saturated ammonium sulfate solution containing 4 mM C-T buffer solution in a cold room. The total yield of aconitase activity was about 90% and the specific activity was increased 3.6 times the original activity of the tissue extract. The ammonium sulfate fraction was dissolved in small quantity of the dialysis solution and dialysed against the same solution overnight in a cold room. The dialysate was centrifuged and the precipitate was descarded. The supernatant obtained was called the "crude enzyme solution".

2. Fractionation on hydroxylapatite: The crude enzyme solution was applied to the top of a hydroxylapatite column, which had been equilibrated with 4 mM C-T buffer solution. The enzyme was eluted at first with 4 mM C-T buffer solution and then with the same solution with NaCl concentration gradient. The absorbance at 280 nm and the aconitase activity of each effluent fraction were measured. The results are shown in Fig. 1. As can be seen from the figure, the crude enzyme solution

contained two protein fractions, both of which showed a citrate dehydrating activity. The total yield of the aconitase activity by this fractionation was 41% : 24% in the first protein peak (fraction nos. 55–70). The specific activities of the first and second peaks were 38 and 290 respectively. The highest specific acitvity was 420 for fraction no. 62 and was 4.1 times that of crude enzyme solution of which specific acitvity was 103. A (nos. 25–35), B (nos. 55–59), and C (nos. 63–69) groups of fractions were concentrated under low pressure with ultrafilter (Sartorius-Membranfilter GmbH) in a cold room and dialyzed against the dialysis solution overnight. The crude enzyme solution and three concentrated fractions, A, B, and C were analyzed by disc electrophoresis. After electrophoresis, the gels were stained for protein fractions and for aconitase activity. As can be seen from the electrophorograms X and X′, the crude enzyme solution showed about 10 protein bands and two aconitase activity bands. Comparing electrophorograms A and X, fraction A obtained by hydroxylapatite chromtography from the curde enzyme solution lost some slower moving protein fraction, and can be seen from electrophorograms A′ and X′, slower moving aconitase acitity band disappeared. As shown in electrophorograms B, B′, C, and C′, fraction B and C obtained by hydroxylapatite chromatography from the crude enzyme solution contained only slower moving protein fractions, and they containd only the slower moving aconitase activity.

3. Fractionation on DEAE-Sephadex: The crude enzyme solution was applied to the top of a DEAE-Sephadex A-50 column which had been equilibrated with 4 mM C-T buffer solution. The elution was carried out with the same buffer solution. The absorbance at 280 nm and the aconitase activity of the effluents were measured. As can be seen from Fig. 2, the crude enzyme solution was separated into four to five protein peaks around the fraction indicated by D, E, F, G, and H. Aconitase activity was detected in the fractions around F, as indicated by dotted line. Aconitase containing fractions (nos. 30–36) were collected and indicated by I. The yield of aconitase activity in the collected fraction I was 40%. The specific activity of this fraction was 860 and 6.1 times that of the crude enzyme solution of which specific acitivity was 142. Fractions D, E, F, G, and H (fraction nos. 23, 26, 32, 41, and 53, respectively) were analyzed by disc electrophoresis and examined for protein and aconitase activity. Each fraction contained a few protein fractions, and the mobilities of the main protein fractions contained in them were in the order of H,G,F,E, and D. Fraction I contained a few protein fractions of which main band corresponded to the aconitase activity band. Fraction I was concentrated and futher separated by rechromatography as described in the next.

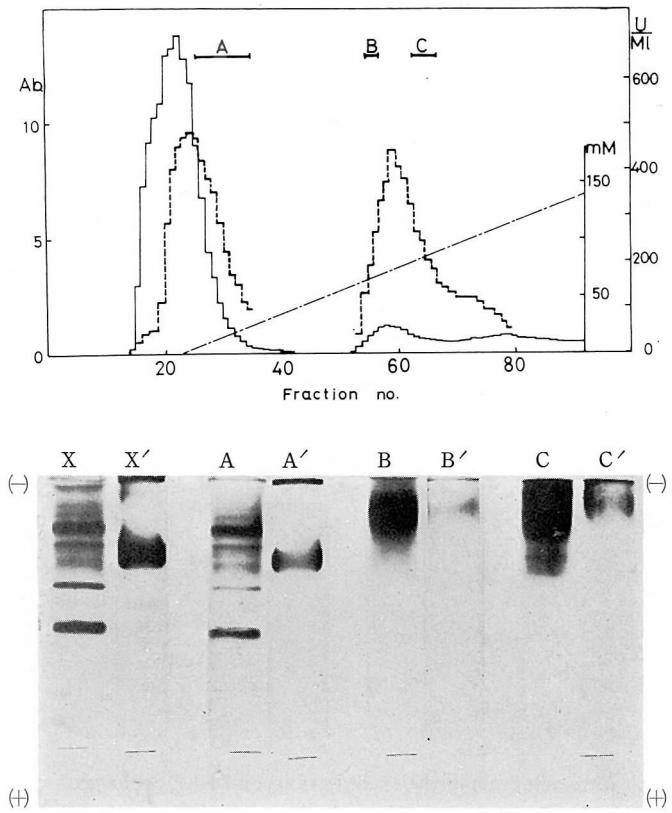

Fig. 1. Chromatogram of the crude enzyme solution on hydroxylapatite.

Thirty ml of a crude enzyme solution (see the text) (7,000U/ml, d=68) were applied on a hydroxylapatite column (3.8×19cm) which had been equilibrated with 4 mM C-T buffer solution (see the text) in a cold room (4°-6°C). The eluents were 4 mM C-T buffer solution. After a few effluents were obtained, 4 mM C-T buffer solution containing on increasing concentration of NaCl was used, as shown by the diagonal dotted line. Ten ml of fractions were collected and analyzed for absorbance at 280nm (——) and for anonitase activity (------) by the method described in the text. Fraction A (fraction nos. 25-35), Fraction B (fraction nos. 55-57), and Fraction C (fraction nos. 65-69) were collected and concentrated separately and analyzed on disc electrophoresis.

X: 0.05 ml of a mixture consisting of 0.3 ml of a crude enzyme solution (7,000U/ml, d=69), 0.5ml of 50% sucrose solution, 0.2ml of 60mM thioglycolate, and 0.2ml of water, were layered on top of the stacking gel. Electrophoresis at 200 V was performed for 2 hrs. in a cold room. After electrophoresis, the gel was stained for protein fraction with Amido Black 10 B and destained electrophoretically. X′: 0.2 ml of the some sample solution were used. After electrophoresis the gel was treated with a Fe^{+2} containing buffer solution and incubated in the aconitase activity staining solution. A: Concentrated Fraction A (6,200U/ml, d=82) was used. Stained with Amido Black. A′: The same solution as A, stained for aconitase activity. B: Concentrated Fraction B (4,200 U/ml, d=12), stained with Amido Black. B′: The same solution as B, stained for aconitase activity. C: Concentrated Fraction C (4,500 U/ml, d=18), stained with Amido Black. C′: The same sample as C, stained for aconitase activity.

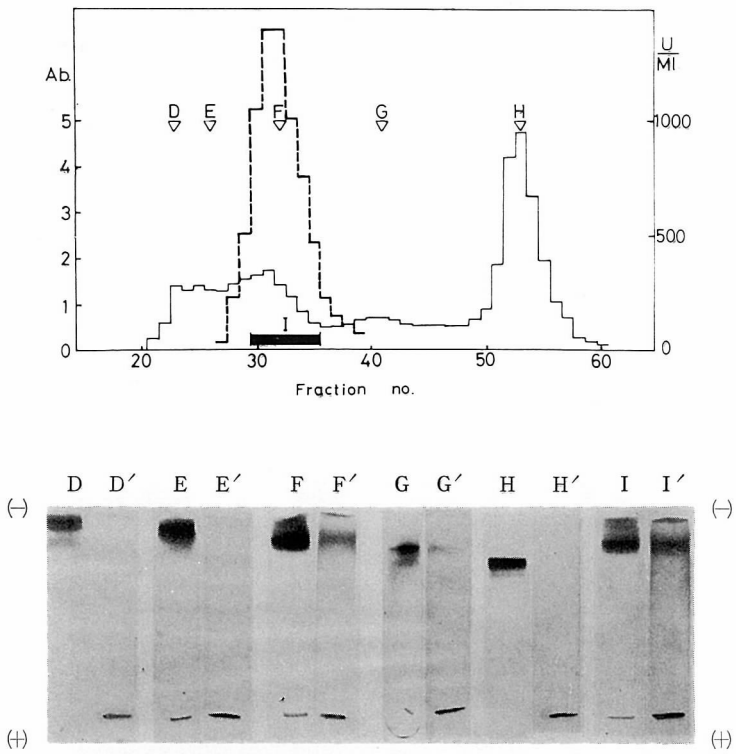

Fig. 2. Chromatogram of the crude enzyme on DEAE-Sephadex.

Thirty ml of a crude enzyme solution (7,500U/ml, d=53) were applied on a DEAE-Sephadex A-50 column (5.0×45 cm) which had been equilibrated with 4 mM C-T buffer solution and eluted with the same buffer solution in a cold room. Fifteen ml fractions were collected and analyzed for the absorbance at 280 nm (——) and for the aconitase activity (------). Fraction D,E,F,G, and H (fraction nos. 23, 26, 32, 41, and 53), and Fraction I (fraction nos. 30-35) were analyzed by disc electrophoresis.

D: 0.1 ml of a mixture consisting of 0.5ml of Fraction D (no activity, d=1.4), 0.5 ml of 50% sucrose solution, and 0.1 ml of 60 mM thioglycolate. Disc electrophoresis at 200 V was performed for 2 hrs. in a cold room. Stained with Amido Black 10 B and destained electrophoretically. D′: The same sample solution as D. Stained by the aconitase activity staining method.

E: Fraction E (35 U/ml, d=1.4), stained with Amido Black. E′: The same sample as E, stained for aconitase activity. F: Fraction F (1,400U/ml, d=1.4), stained for protein.

F′: The same sample as F, stained for aconitase activity. G: Fraction G (50 U/ml, d=0.7) stained for protein. G′: The same sample as G, stained for aconitase activity. H: Fraction H (no activity, d=4.6), stained for protein. H′: The same sample as H, stained for aconitase activity. I: Fraction I (1,000 U/ml. d=1.2), stained for protein. I′: The same sample as I, stained for aconitase activity.

4. Fractionation by rechromatography on CM-Sephadex:
The concentrated fraction I was applied to the top of a CM-Sephadex C-50 column which had been equilibrated with 4 mM C-T buffer solution. The absorbance and the aconitase activity of each effluent were measured. As can be seen from the results shown in Fig. 3, three protein fractions were separated and the main protein fraction L corresponded with the aconitase activity. The yield of aconitase activity in the collected fraction including nos. 19–24 was 27%. The specific activity of this fraction was 940 and was 1.4 times that of the applied enzyme solution, of which specific activity was 667. Fraction nos. 9 (J), 15 (K), and 21 (L) were analyzed by disc electrophoresis. Only fraction no. 21 contained aconitase activity and only one protein bond, which corresponeded to each other.

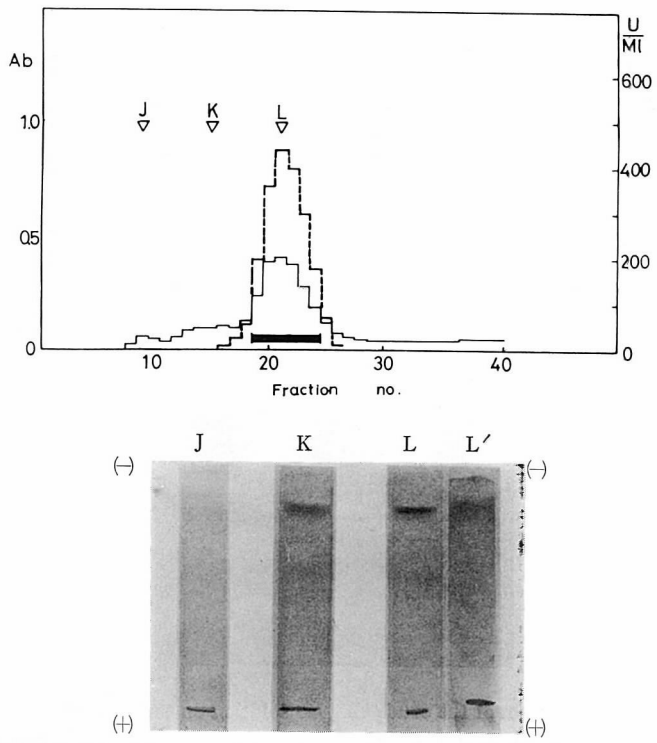

Fig. 3. Recromatography on CM-Sephadex of the aconitase containing fraction obtained by DEAE-Sephadex chromatography.

5.5 ml of the concentrated Fraction I (12,000U/ml, d=18), obtained by column chromatography on DEAE-Sephadex as shown in Fig. 2, were applied on a CM-Sephadex column (2.5×35 cm) which had been equilibrated with 4 mM C-T buffer solution and eluted with the same buffer solution. Ten ml fractions were collected and analyzed for absorbance at 280 nm (——) and for aconitase activity (------). Fraction no. 9, 15, and 21 were analyzed by disc electrophoresis, and indicated by J, K, and L, respectively.
J: Fraction no. 9 (no activity, d=0.06) stained with Amido Black 10 B. K: Fraction no. 15 (no activity, d=0.10), stained with Amido Black. L: Fraction no. 21 (440 U/ml, d=0.41), stained with Amido Black. L′: The same sample as L, stained for aconitase activity.

5. Fraction on CM-Sephadex: On the other hand, the crude enzyme solution was applied on a CM-Sephadex C-50 column which had been equilibrated with 4 mM C-T buffer solution. The absorbance and the aconitase activity of each eluate obtained with the same buffer solution were measured. As can be seen from the results shown in Fig. 4, the crude enzyme solution was separated into two protein peaks. The first peak around fraction no. 8 contained the larger part of protein but the aconitase activity was mainly contained in the slower fractions around fraction no. 17. The total yield of aconitase activity was 44% : 11% in the first fractions (nos. 8–12) and 33% in the second fractions (nos. 13–20).

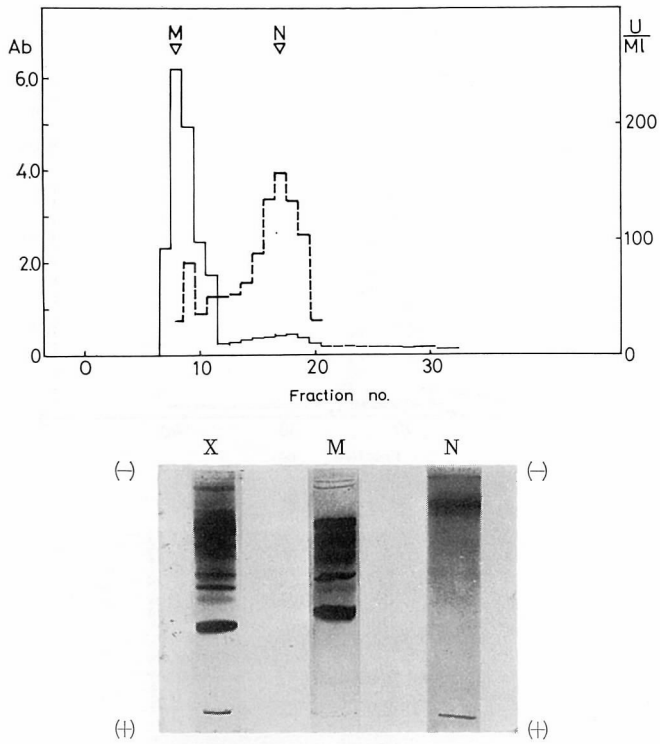

Fig. 4. Chromatogram of the crude enzyme solution on CM-Sephadex.

Five ml of a crude enzyme solution (4,500 U/ml, d=46) were applied on a CM-Sephadex C-50 column (2.5×40 cm) which had been equilibrated with 4 mM C-T buffer solution and eluted with the same solution in a cold room. Ten ml of fractions were collected and analyzed for absorbance at 280 nm (——) and for aconitase activity (------).

X: 0.05 ml of a mixture containing 0.1 ml of a crude enzyme solution (4,500U/ml, d=46), 0.5ml of 50% sucrose solution, 0.1 ml of 60 mM thioglycolate, and 0.3 ml of water were layered on top of stacking gel. Electrophoresis at 200 V was performed for 2 hrs. in a cold room. The gel was stained with Amido Black 10 B. M: Fraction no. 8 (45U/ml, d=6.2), stained with Amido Black. N: Fraction no. 18 (130U/ml, d=0.4), stained with Amido Black.

The specific activity of the second fractions was 260 and was 2.6 times that of the crude enzyme solution, of which specific activity was 98. Fraction no. 8, 17 and the crude enzyme solution were analysed by disc electrophoresis. The crude enzyme solution showed about 10 protein bands, fracton no. 8 lost some of them, and fraction no. 17 showed only a few slower moving bands.

6. Fractionation of aconitase containing fractions obtained by DEAD-Sephadex chromatography by rechromatography on hydroxylapatite:
The aconitase containing fraction obtained by column chromatography on DEAE-Sephadex in a similar manner as discribed above were combined and concentrated under low pressure and applied to the top of a hydroxylapatite column which had been equilibrated with 4 mM C-T buffer solution.

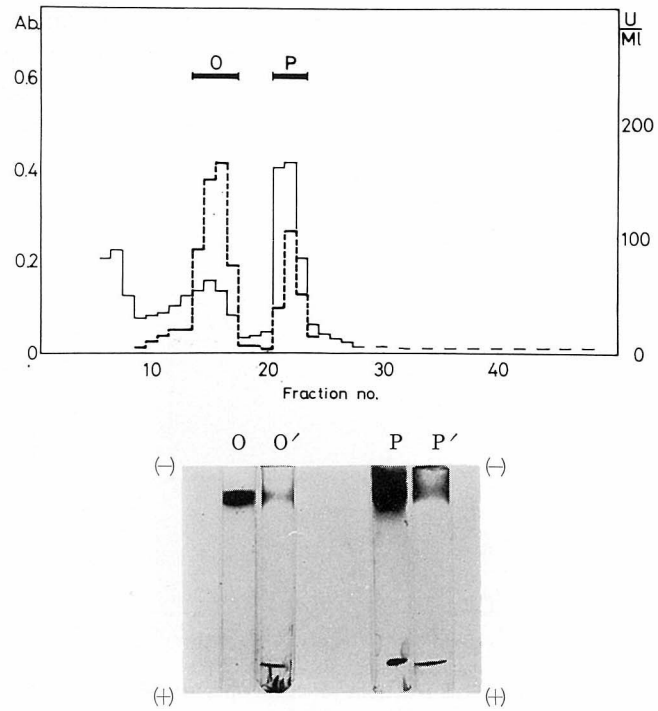

Fig. 5. Rechromatography of the aconitase containing fraction obtained by DEAE-Sephadex chromatography on hydroxylapatite.

Aconitase activity containing fractions obtained by column chromatography on DEAD-Sepadex were combined and cocentrated under low pressure. 1.2 ml of concentrate (5,000 U/ml, d=7.5) were applied to a hydroxylapatite column (1.6×10 cm) which had been equilibrated with 4 mM C-T buffer solution. The eluent at first was 4 mM C-T buffer solution and at fraction no. 10 the eluent was changed to the buffer containing 200 mM NaCl. Three ml fractions were collected and analyzed for absorbance at 280 nm (——) and for aconitase activity (------). Fraction nos. 14-16 (O) and 21-32 (P) were collected separately, concentrated under low pressure, and analyzed by disc electrophoresis.
O: Concentrated fraction nos. 14-17 (800 U/ml, d=0.9), stained with Amido Black 10 B.
O′: the same sample as O, stained for aconitase activity. P: Concentrated fraction nos. 21-32 (610 U/ml, d=2.0), stained with Amido Black. P′: The same sample as P, Stained for aconitase activity.

The enzyme was eluted at first with the buffer and then with the buffer solution containing 200 mM NaCl. The absorbance at 280 nm and the aconitase activity of each effluent were measured. As can be seen from the reults shown in Fig. 5, three protein peaks appeared, of which two contained aconitase activity. The yield of aconitase activity was 20% in the second peak (O) (fraction nos. 14–17) and 8% in the third peak (P) (nos. 21–23). Fractions O and P were concentrated under low pressure and analyzed by disc electrophoresis. As can be seen from the electrophorograms fraction O was fairly homogeneous and the aconitase activity band corresponded to the protein band. Fraction P showed a few protein bands, of which the main protein fraction contained aconitase activity.

7. Fractionation by acrylamide gel electrophoresis: The crude enzyme solution was separated by disc electrophoresis, in a glass tube of 15×70mm. After electrophoresis the gel was cut into 2 mm sections and each section was put into a cellophane tube with the dialysis solution and dialyzed against the same solution (as described in the section "Materials and Methods"). After the dialysis the aconitase activity of the dialyzed solution

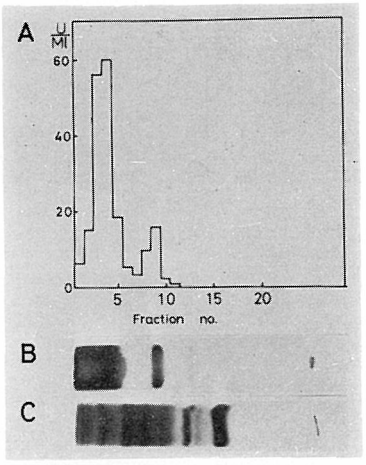

Fig. 6. Aconitase activity of the fractions of crude enzyme solution separated by disc electrophoresis.

A: One ml of the mixture containing 0.6 ml of a crude enzyme solution (4,500 U/ml, d=40), 0.4 ml of 50 % sucrose solution and 0.2 ml of 60 mM thioglycolate was applied on top of the stacking gel. Disc electrophoresis was carried out in a glass tube of 15×70 mm at 200 V for 2 hrs in a cold room. After electrophoresis, the gel was cut into 2 mm sections. Each section was dialyzed against the dialysis solution, then against 4 mM C-T buffer solution, as described in the section of method. The activity of each dialysate was measured and plotted.

B: A crude enzyme solution (7,500 U/ml, d=53) run on disc electrophoresis. Stained for aconitase activity.

C: The same sample solution as B, stained with Amido Black 10 B.

was measured. As can be seen from the results shown in Fig 6, crude enzyme solution showed two peaks of aconitase activity and the total aconitase activities recovered were 15% in fraction nos. 1-6 and 3 % in fraction nos. 7-11.

DISCUSSION

Aconitase isozymes: Koen and Coodman[12] reported recently on two isozymes of aconitase, cytoplasmic and mitochondrial forms. They investigated the tissue distribution of the isozymes in mice and Slow loris using starch gel electrophoresis. Comparing our results with their report, faster moving aconitase fraction by disc electrophoresis and faster eluting aconitase fraction of the hydroxylapatite column as shown in Fig 1 and 6, may be the cytoplasmic form described by Koen and Coodman[12]. On the other hand, the mitochondrial form would correspond to the aconitase fraction moving more slowly on disc electrophoresis and eluted slowly on the hydroxylapatite column. This aconitase fraction seem to correspond to that obtained by the DEAE-Sephadex A-50 column as shown in Fig. 2. The purified aconitase obtained by rechromatography on hydroxylapatite and CM-Sephadex shown in Fig 3 and 5 may also be the mitochondrial form.

Comparison of the fast and the slowly eluted aconitase fractions on the hydroxylapatite column: The two fractions containing aconitase activity obtained by hydroxylapatite column chromatography were separately fractionated with ammonium sulfate, dialyzed and fractionated by Sephadex G-200 column. The results are shown in Fig 7. Fig. 7-A represents the chromatogram of first fraction and 7-B shows the chromatogram of second fraction. The elution volumes of both aconitase fractions shown in Fig. 7-A and B are equal, when the same column was used. This suggests that the molecular weight of the two aconitase fractions obtained by hydroxylapatite chromatography may be identical. Studies on the molecular weight and other properties of aconitase isozymes are now under way.

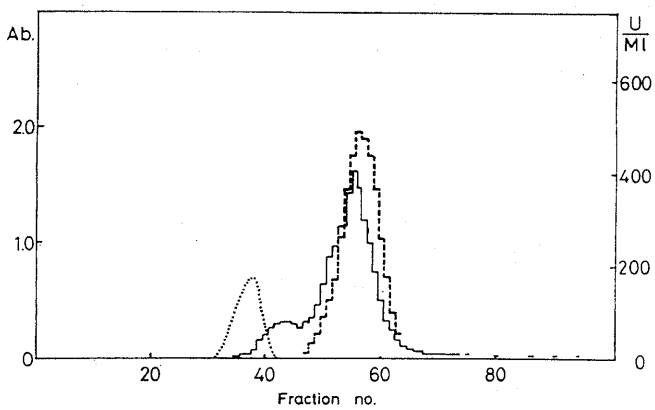

Fig. 7-A.

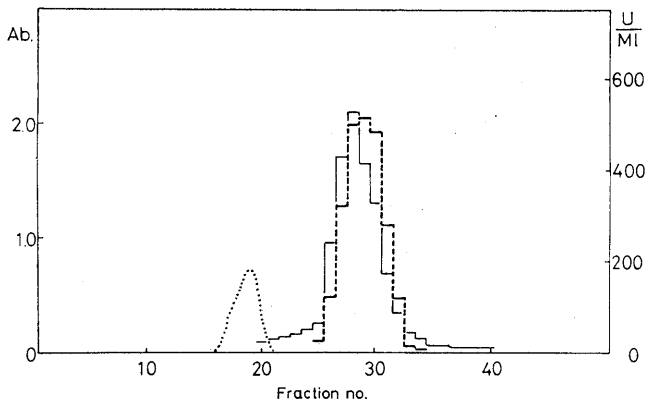

Fig. 7-B.

Fig. 7. Rechromatography of the aconitase containing fraction obtained by hydroxylapatite chromatography on Sephadex G-200.

A: The first fractions containing aconitase activity obtained by column chromatography on hydroxylapatite were combined. Fraction obtained by ammonium sulfate fractionation (60-70 % saturation) was dialyzed. Seven ml of the dialyzate (10,000U / ml, d=38) were applied to a Sephadex G-200 column (2.5 × 93 cm) which had been equilibrated with 4 mM C-T buffer and eluted with the same buffer solution. Ten ml of fractions were collected and analyzed for absorbance at 280 nm (——) and for aconitase activity (------). B: The second fractions containing aconitase activity obtained on hydroxylapatite. Fraction obtained by ammonium sulfate fractionation (60-70 % saturation) was dialyzed. One ml of the dialyzate (27,000 U/ml, d=80) was applied to the Sephadex G-200 column (2.5 × 93 cm). Five ml fractions were collected and analyzed for absorbance (——) and for activity (------). One ml of 1 % Blue Dextran 2000 solution was applied to the top of the column to measure the void vilume (·····).

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SUMMARY

The aconitase activity staining method on the polyacrylamide gel disc electrophoresis was devised and applied to the detection of aconitase.

Aconitase of swine heart was purified by ammonium sulfate fractionation and column chromatographies. By chromatography on hydroxylapatite or by polyacrylamide gel electrophoresis, the ammoniun sulfate fraction of swine heart aconitase was separated into two forms. The elution volumes of both aconitase fractions were equal by column chromatography on Sephadex G-200.

One form of aconitase of ammonium sulfate fraction was obtained by column chromatography on DEAE-Sephadex A-50. This form was rechromatographed on CM-Sephadex C-50 and was found to be fairly homogeneous by disc electrophoresis.

Zusammenfassung

Eine Farbungsmethode der Akonitase-Aktivität in dem Disk-Elektrophorese-Gel wurde ausgearbeitet und zur Sichtbarmachung der Akonitase angewandt.

Die Akonitase des Schweinherzens wurde durch Ammoniumsulfat fraktioniert und mit Säulenchromatographien gereinigt. Durch die Chromatographie auf Hydroxylapatit oder durch Polyacrylamidgel-Elektrophorese wurde die Akonitase aus Schweinherzen in zwei Formen getrennt. Die Elutionsvolumen der beiden Akonitase-Fraktionen waren gleich auf Säulenchromatographien mit Sephadex G-200.

Die Fraktion durch Ammoniumsulfat zeigte nur eine Form von Akonitase auf Säulenchromatographie von DEAE-Sephadex A-50. Die erworbene Fraktion wurde mit CM-Sephadex C-50 rechromatographiert und gefunden auf Disk-Elektrophorese fast homogen zu sein.

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