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## STARCH-GEL ELECTROPHORESIS OF CITRATE-CONDENSING ENZYME FROM PIG HEART

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## SUMMARY

Citrate-condensing enzyme from pig heart can exist *in vitro* as two distinct species which are separable by starch-gel electrophoresis. Several mild types of treatment can interconvert these enzymes and suggest that the separate forms arise in the process of purification; the two enzymes may differ only in the state of reduction of their sulphydryl groups.

## INTRODUCTION

Crystalline citrate-condensing enzyme from pig heart has been shown to be a homogeneous protein by ultracentrifugal and electrophoretic techniques<sup>1,2</sup>. In our recent work with this protein we have applied immunological and starch-gel electrophoretic methods to the study of its homogeneity. Although immunochemical studies<sup>3</sup> gave no information on this question, the results reported in this paper indicate that pig-heart citrate-condensing enzyme can exist in two closely related forms that are distinguishable by starch-gel electrophoresis.

## METHODS AND MATERIALS

Citrate-condensing enzyme was prepared from pig heart and its enzymic activity assayed spectrophotometrically at 340 m $\mu$  using the coupled malate dehydrogenase system<sup>2</sup>. Boehringer malate dehydrogenase and CoA were used and acetyl-CoA was prepared by the method of SIMON AND SHEMIN<sup>4</sup>. Protein was determined by the method of LOWRY *et al.*<sup>5</sup>. Enzyme solutions were ultracentrifuged in a Spinco Model E Ultracentrifuge at 59680 rev./min. Chromatography on DEAE-cellulose was carried out as described previously<sup>3</sup> using either a stepwise elution or a gradient elution method.

Zone electrophoresis in starch gel was carried out at 4° and at pH 8.6 using 0.08 M Tris-citrate in the gels and 0.3 M sodium borate in the electrode chambers<sup>6,7</sup>. The gel molds measured 17.5 cm  $\times$  1.8 cm  $\times$  0.6 cm. The enzyme sample (80–120 units/ml) was dialyzed against the Tris-citrate buffer and 0.05–0.1 ml applied to a piece of Whatman No. 17 chromatography paper for insertion into the gel. A potential difference of approx. 4.5 V/cm was applied to the gels and electrophoresis allowed to

proceed for 14–16 h. The gels were then cut horizontally and the top half stained with a saturated amido black solution; the bottom half was sliced into 25–30 sections with a special cutting apparatus which enabled reproducible cutting of sections as short as 0.25 cm. Each section was placed in a tube and stored at  $-20^{\circ}$ .

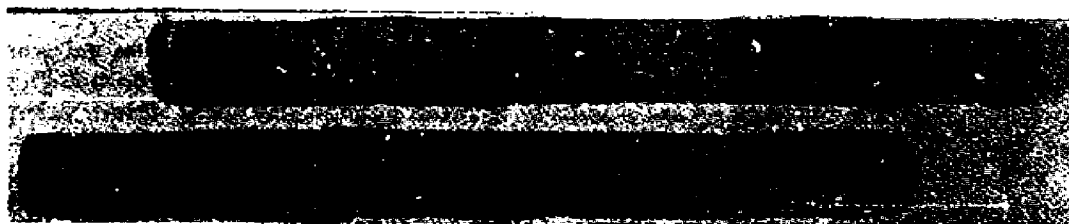


Fig. 1. Starch gels stained for protein, following electrophoresis of two separate preparations of three times recrystallized citrate-condensing enzyme.

The fractions were thawed and eluted by crushing the gel in 0.08 M Tris-citrate buffer (pH 8.6); 0.5 ml of buffer was added for each 0.25 cm length of gel. Enzyme activity was measured on an aliquot of the supernatant solution. A 60–65% recovery of the initial enzymic activity was usually obtained. Electrophoresis with different buffer systems at other pH values gave poorer resolution. The enzyme migrated towards the positive electrode.

#### RESULTS

Fig. 1 shows the protein-stained starch gels from two separate preparations of three times recrystallized citrate-condensing enzyme. The lower gel shows two bands that

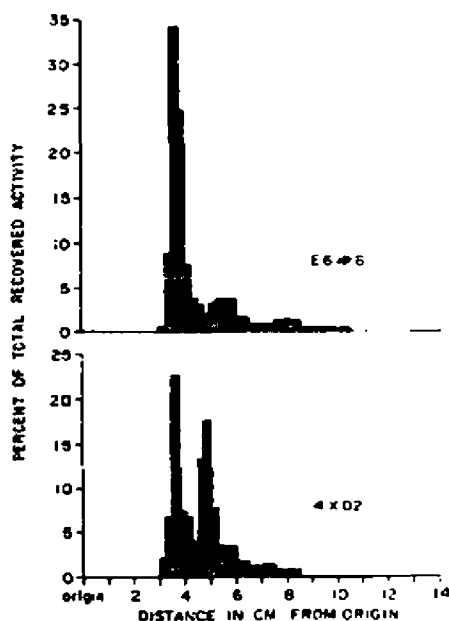


Fig. 2. Enzyme elution patterns obtained from gels shown in Fig. 1. The relative positions of the bands staining for protein are marked in cross-hatching along the horizontal ordinate.

stain as protein. Only one of these bands can be seen in the photograph of the upper gel. The original specimen of the upper gel, however, showed a second faint band corresponding to the second band of the lower gel. The enzyme elution patterns obtained from these gels (Fig. 2) show that each of these bands corresponds to a separate peak of enzyme activity. In the upper gel, there is a small peak of activity which corresponds to the faintly stained band.

The specific activities of both forms of the enzyme eluted from the gel were approximately equal. Both electrophoretic forms had identical immunological specificity<sup>2</sup>. When immune serum was fully titrated against one form, there was no residual capacity to react subsequently with the second.

Analytical ultracentrifugation was carried out on a twice recrystallized preparation which on electrophoresis demonstrated two distinct activity peaks. 400 units of enzyme (13 mg) in 0.7 ml of 0.3 M sodium borate (pH 8.6) were centrifuged at 59 680 rev./min and 20° for 1.5 h. At 38, 52 and 86 min (Fig. 3) a single peak with minimal asymmetry was seen.

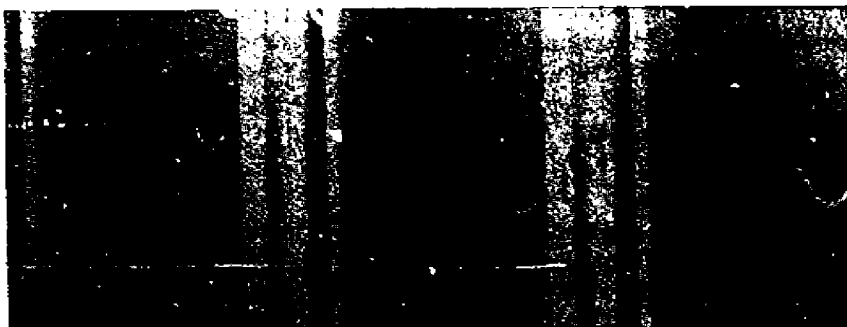


Fig. 3. The sedimentation pattern at 38, 52 and 86 min of a twice recrystallized enzyme preparation which on starch-gel electrophoresis showed 2 distinct regions of enzyme activity.

The gel sections containing each of the two enzymes were separately inserted into new gels for re-electrophoresis (Fig. 4). Each form yielded only a single band of enzyme activity with the same mobility as the original.

Enzyme solutions were obtained at five successive steps during the purification of citrate-condensing enzyme<sup>2</sup>: (a) original extract of pig heart, (b) protein soluble in a 50% ammonium sulfate solution, (c) protein that was precipitated in a 70% ammonium sulfate solution (50–70% ammonium sulfate), (d) protein that was adsorbed and eluted from DEAE-cellulose, (e) crystalline enzyme obtained from ammonium sulfate solutions. It was necessary to concentrate the solutions obtained at steps a and b so that sufficient enzyme activity could be placed on the gel for analysis. We failed to obtain clear resolution of protein or of enzyme activity from these solutions because of their high protein concentrations.

Three preparations of the 50–70% ammonium sulfate precipitate (step c) showed 2 citrate-condensing enzymes and three others showed only a single enzyme. In those preparations showing a single enzyme, its migration rate corresponded to the more rapidly migrating form of those with 2 enzymes. When a preparation with two enzymes was re-dialyzed and again studied by starch-gel electrophoresis, the

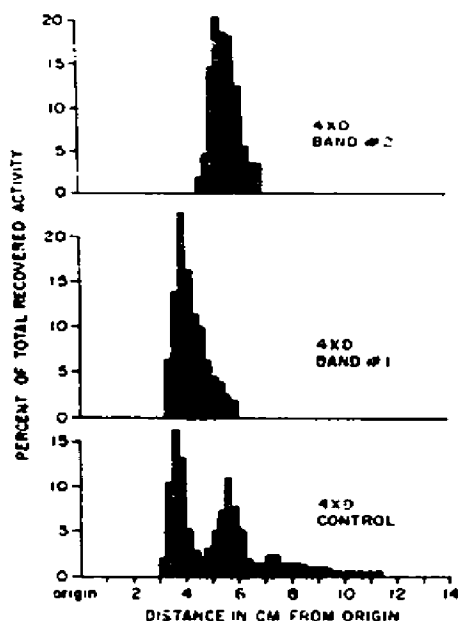


Fig. 4. Enzyme elution patterns obtained from the individual enzyme bands which were excised from their original gel and re-electrophoresed separately. The lower pattern is from a sample of the original preparation and was run concurrently.

more slowly migrating enzyme was greatly diminished or absent with a corresponding increase in the rapid enzyme (Fig. 5); there was no loss of total activity. This behavior appeared to be caused by the prolonged dialysis rather than altered buffer composition, for when a preparation altered by re-dialysis was further dialyzed against the original buffer, the slower migrating enzyme was not restored. However, preparations at this state of purification which had always shown only the more rapidly migrating enzyme had not been dialyzed longer than the others.

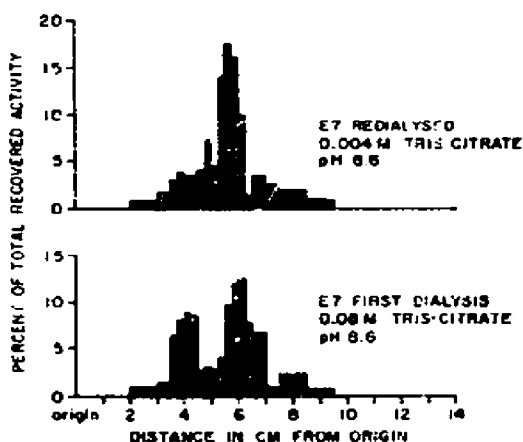


Fig. 5. Enzyme elution patterns of an enzyme preparation at the end of step c. The lower pattern was obtained after the initial dialysis and the upper one after a second dialysis.

Chromatography on DEAE-cellulose caused conversion of the faster to the slower migrating electrophoretic form of enzyme (Fig. 6). In 2 other experiments of this type, almost all of the enzyme was converted to the slowly moving form; prior to chromatography one of these showed 2 peaks and the other only a single rapidly migrating one. Since the recovery of enzyme from the DEAE-cellulose in these

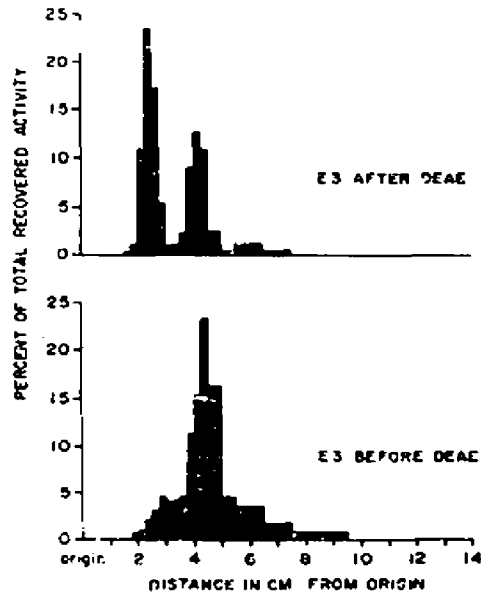


Fig. 6. Enzyme elution patterns on an enzyme preparation before and after step d.

experiments was approx. 80%, it is not possible to explain the alteration in the amounts of the 2 enzymes on the basis of loss of one form. The enzyme was concentrated for electrophoresis either by vacuum evaporation at 30° or adsorption on calcium phosphate gel and elution in a small volume of 50% ammonium sulphate<sup>3</sup>.

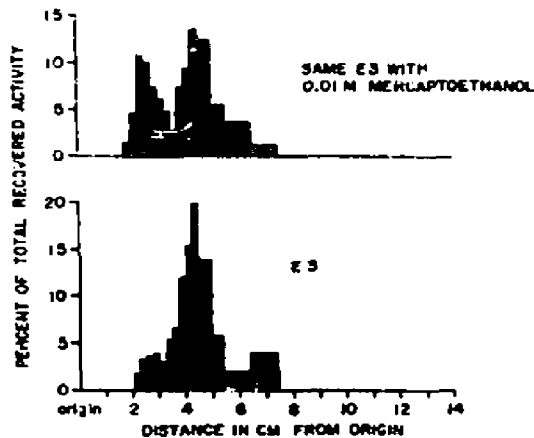


Fig. 7. Enzyme elution patterns of an enzyme preparation before and after treatment with mercaptoethanol.

The electrophoretic behavior was similar after both concentrating procedures. The electrophoretic behavior following chromatography was not influenced by the presence of the vacuum-concentrated eluates which contained protein but no enzyme; it is unlikely therefore that some other protein influenced the electrophoretic behavior of the enzyme.

Three preparations were followed through 4 recrystallization steps and showed no further changes in electrophoretic behavior.

Further studies were carried out on the preparations which had been purified to the end of the third step. When one of these which had always shown only a single rapidly migrating enzyme was placed in 0.01 M mercaptoethanol, subsequent electrophoresis showed that the slower migrating enzyme had been formed (Fig. 7). In preparations with the 2 enzyme forms in which the slower one decreased after dialysis, a restoration of the slower enzyme could be accomplished with mercaptoethanol.

#### DISCUSSION

The presence of multiple protein forms for a single enzyme activity in a single tissue is well established. Possible explanations for such multiple enzyme forms are: (a) the proteins may be totally different, (b) one or both of the proteins may have multiple activities, (c) the proteins may be interconvertible, *i.e.* one is a slight chemical modification of the other.

The results obtained by us indicate that the citrate-condensing enzyme from pig heart can exist in two closely related forms. Several mild types of treatment can interconvert these enzymes, suggesting that the separate forms may arise in the process of purification. There are several enzymes<sup>9-12</sup> which seem to be similar to the condensing enzyme in this respect.

It is unlikely that the two enzymes represent two different states of association since only a single peak is seen upon ultracentrifugation.

Citrate-condensing enzyme contains 2-3 moles of sulfhydryl groups that are not essential for its activity<sup>3</sup>. The action of mercaptoethanol in causing the change of one form to the other, suggests that the modification involves an SH to SS conversion. The slowly migrating form would then be SH enzyme and the more rapidly moving form the disulfide enzyme; dialysis would lead to the oxidation of SH groups to SS and chromatography on DEAE-cellulose would cause a change from the SS enzyme to the reduced enzyme. Such a conversion on DEAE-cellulose is not expected, but cannot be excluded.

The findings do not eliminate the possibility that citrate-condensing enzyme may exist as more than a single molecular species in living pig-heart tissue.

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