# Isolation of Fructose Diphosphate Aldolases A, B, and C*

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ABSTRACT: Three distinct fructose diphosphate aldolases termed A, B, and C have been detected in mammalian tissues. Methods are presented which allow the isolation of these three enzymes from rabbit muscle, liver, and brain, respectively. The most significant common feature in the methods of preparation is selective elution of the enzymes from cellulose phosphate columns with the substrate fructose diphosphate. The enzymes isolated by these techniques have high specific activities and appear to be homogeneous by the criteria applied.

Three distinct fructose diphosphate aldolases termed aldolases A, B, and C (Herbert *et al.*, 1940; Warburg and Christian, 1943; Peanasky and Lardy, 1958; Rutter, 1964; Penhoet *et al.*, 1966) have been detected in mammalian tissues. In addition, hybrid enzymes composed of the subunits of these parental types have been produced *in vitro* by reversible dissociation of binary mixtures of parental aldolases and have been detected *in vivo* in tissues containing more than one of these subunit types (Penhoet *et al.*, 1966, 1967; Rutter *et al.*, 1968; Lebherz and Rutter, 1969; Christian *et al.*, 1966; Herskovitz *et al.*, 1967; Matsushima *et al.*, 1968a, b; Foxwell *et al.*, 1966; Anstall *et al.*, 1966; Pietruszko and Baron, 1967). Aldolase A has been isolated from skeletal muscle of a number of organisms (Rensing *et al.*, 1967; Kwon and Olcott, 1965; Shibata, 1958; Schwartz and Horecker, 1966). Aldolase B has been isolated from bovine (Peanasky and Lardy, 1958) and rat and rabbit liver (Rajkumar *et al.*, 1966; Matsushima *et al.*, 1968b). We report here procedures for the isolation of aldolases A and B from rabbit muscle and liver, respectively, and aldolase C and members of the aldolase A–C hybrid set from rabbit brain. The most significant common feature in the methods of preparation is selective elution of the enzymes from cellulose phosphate chromatographic columns with the substrate FDP, a modification of the method originally utilized by Pogell (1962). The methods are rapid and reproducible, yielding enzymes of high specific activity and molecular homogeneity. With slight modifications, the isolation procedures may be applicable to the isolation of class I aldolases from a wide variety of biological systems.

## Materials[1]

Rabbit muscle and livers were removed from freshly killed rabbits obtained from Lab Associates, Inc., Kirkland, Wash. Frozen rabbit brains were procured from Pel-Freez Biologicals, Inc., Rogers, Ark., or Lab Associates, Inc.

Phosphocellulose (0.9 mequiv/g) was obtained from Schleicher & Schuell, Inc., Keene, N. H. DEAE-Sephadex A-50 (3.5 mequiv/g, 40–120 $\mu$ particle size) and other Sephadex preparations were obtained from Pharmacia Fine Chemicals, Piscataway, N. Y. Enzyme grade ammonium sulfate obtained from Mann Research Laboratories, Inc., New York, N. Y., was employed in all studies. Cellulose acetate electrophoresis strips were purchased from the Gelman Co., Ann Arbor, Mich.

Glycerol phosphate dehydrogenase–triosephosphate isomerase mixture was procured from the California Corp. for Biochemical Research. All other biochemical reagents were obtained from the Sigma Chemical Co.

## Methods

Aldolase assays were performed at 25° in a Gilford recording spectrophotometer as described by Blostein and Rutter (1963), except that 0.1 M Tris-Cl (pH 7.5) was substituted for the glycylglycine buffer. The protein in crude homogenates and solutions was determined by the spectrophotometric method of Warburg and Christian (1942). Concentrations of purified aldolases were also determined spectrophotometrically using extinction coefficients of 0.91, 0.89, and 0.88 for aldolases A, B, and C, respectively (Penhoet *et al.*, 1966).

All operations in the purification procedures were performed at 4° unless otherwise noted. Contact of all aldolase preparations with FDP was minimized since it has been demonstrated (Woodfin, 1967; Lai *et al.*, 1968) that the enzymes are subject to slow inactivation by substrate.

The procedure for cellulose acetate electrophoresis and detection of aldolase activity has been described previously (Penhoet *et al.*, 1966). Disc gel electrophoresis was performed according to Davis (1964) using Tris-Cl buffer (pH 8.7). Proteins were detected by staining with Amido Black 9B or coomassie blue stains. Dilute protein solutions were concentrated under nitrogen using an Amicon ultrafiltration apparatus with UM-1 membranes.

## Results

*Preparation of Aldolase A.* A summary of the purification procedure is described in Table I; details are as follows.

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[1] Abbreviation used is: PMSF, phenylmethanesulfonyl fluoride.

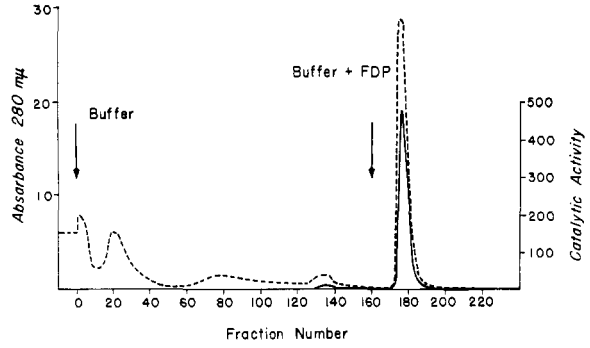


FIGURE 1: Phosphocellulose chromatography of rabbit muscle aldolase. The 45–60% ammonium sulfate fractionation obtained as described in the text and equilibrated with 0.01 M Tris-Cl–0.001 M EDTA (pH 7.5) was applied to a 3.7 × 100 cm phosphocellulose column equilibrated with the same buffer. After the sample was applied, the column was washed with 0.05 M Tris-Cl–0.005 M EDTA (pH 7.5) until the red hemoglobin band was removed and the absorbance of the effluent at 280 mμ fell below 0.1. The aldolase fraction was then eluted with the same buffer to which had been added 0.0025 M FDP. The volume of the individual fractions collected was 10 ml.

CRUDE EXTRACT. Rabbits were anesthetized with nembutal (30 mg/kg); the skeletal muscles were quickly removed, cooled in ice, and ground in a meat grinder. The minced muscle was then suspended in three volumes (v/w) of 0.05 M Tris-Cl–0.005 M EDTA–0.004 M β-mercaptoethanol (pH 7.5). The suspension was homogenized in a commercial blender at low speed for 60 sec and then centrifuged at 14,000*g* for 1 hr.

AMMONIUM SULFATE FRACTIONATION. The opalescent supernatant was brought to 45% saturation by the addition (over a 2-hr period) of 278 g of ammonium sulfate/l. of extract. After standing for 1 hr, the solution was centrifuged at 14,000*g* for 1 hr. The precipitate was discarded and the resulting supernatant was brought to 60% ammonium sulfate saturation by the addition of 98 g of ammonium sulfate/l. of supernatant. The pH then was adjusted to 7.5 with 6 N $NH_4OH$ and the solution was allowed to stand at least 2 hr before centrifugation at 14,000*g* for 1 hr. The 45–60% ammonium sulfate precipitate was dissolved in 0.01 M Tris-Cl–0.001 M EDTA (pH 7.5); after the protein concentration was adjusted to 40 mg/ml, the solution was passed over a Sephadex G-25 (coarse) column (4.5 × 120 cm) equilibrated with the same buffer.

PHOSPHOCELLULOSE CHROMATOGRAPHY. The desalted 45–60% ammonium sulfate fraction (approximately 300 ml) was applied to a 3.7 × 100 cm cellulose phosphate column, equilibrated with 0.01 M Tris-Cl–0.001 M EDTA (pH 7.5).

The column elution pattern is presented in Figure 1. The column was first washed with 2 l. of 0.05 M Tris-Cl–0.005 M EDTA (pH 7.5) until the narrow red (hemoglobin) band was eluted and the optical density of the effluent at 280 mμ dropped below 0.1. Substrate elution was then carried out with 1000 ml of 0.0025 M FDP–0.05 M Tris-Cl–0.005 M EDTA (pH 7.5). The aldolase A was eluted sharply with a maximum specific activity of approximately 17 μM FDP cleaved/min per mg.

CRYSTALLIZATION. The peak fractions obtained from phosphocellulose chromatography were dialyzed against a final concentration of 50% saturated ammonium sulfate in 0.01 M Tris-Cl–0.001 M EDTA (pH 7.5). Crystals formed overnight were collected by centrifugation at 35,000*g* for 30 min. A typical crystal crystalline preparation is shown in Figure 2. The aldolase crystals were stored for extended periods of time (up to 6 months) as suspensions in 50% saturated ammonium sulfate solutions with little loss of activity.

TABLE I: Purification of Aldolase A from Rabbit Muscle.

| Purifcn Steps | Total Act. | Total Protein (g) | Sp Act. | % Yield |
|---|---|---|---|---|
| 1. 14,000*g* supernatant of crude extract (from 1 kg of muscle) | 78,700 | 60.26 | 0.13 | 100 |
| 2. 45–60% $(NH_4)_2SO_4$ precipitate | 55,900 | 11.8 | 4.8–5.2 | 71 |
| 3. Phosphocellulose chromatography | 54,400 | 3.2 | 17 | 69 |
| 4. Crystallization | 46,500 | 3 | 15–16 | 59 |

The specific activity of different preparations of aldolase A varied from 14 to 18.

*Preparation of Aldolase B.* A summary of the isolation procedure for aldolase B is presented in Table II. With the following exceptions, it is purified in the manner described above for aldolase A. CRUDE EXTRACT. Livers were removed from fully fed, freshly killed rabbits (frozen rabbit livers should not be employed since the aldolase is apparently less stable in extracts from this source), homogenized in two volumes of cold 0.001 M Tris-Cl–0.001 M EDTA (pH 7.5) for 60 sec, and centrifuged at 37,500*g* for 60 min. The supernatant was then passed through Whatman No. 1 filter paper to remove lipid material.

AMMONIUM SULFATE FRACTIONATION. A 45–60% ammonium sulfate fraction was obtained using 0° saturated ammonium sulfate. This fraction was desalted and resolved on phosphocellulose as described for aldolase A.[2]

CRYSTALLIZATION. Aldolase B (approximately 10 mg/ml) was dialyzed against 10% saturated ammonium sulfate (53 g/l.)–0.001 M EDTA (pH 7.5) for 2 hr. The ammonium sulfate concentration was then raised slowly to 55% by adding saturated ammonium sulfate to the dialysis vessel over a period of several hours. This procedure produced homogeneous-appearing, needle-type crystals as shown in Figure 2.

The specific activities of aldolase B preparations have varied from 1 to 2. Specific activities closer to 2 can be expected on carrying out the procedure expeditiously.

*Preparation of Aldolase C and the A–C Hybrids.* The preparation of aldolase C and the A–C hybrids has been outlined previously (Penhoet *et al.*, 1966; Rutter *et al.*, 1968).

The fractionation procedure is summarized in Table III;

[2] For preparation of small quantities of aldolases A, B, or C, the ammonium sulfate fractionation step may be deleted. In this case, the supernatant from step I is equilibrated with 0.01 M Tris-Cl–0.001 M EDTA (pH 7.5) by Sephadex G-25 chromatography. The resultant solution is then applied directly to the cellulose phosphate column. Inclusion of the ammonium sulfate step substantially reduces the volumes applied to the phosphocellulose columns and results in purer end products, particularly in the case of aldolase B.

TABLE II: Purification of Aldolase B from Rabbit Liver.

| Fraction | Vol (ml) | Act. (μmoles of FDP/min per ml) | Protein (mg/ml) | Total Protein (mg) | Total Act. | Sp Act. | % Yield |
|---|---|---|---|---|---|---|---|
| Without ammonium sulfate fractionation | | | | | | | |
| 37,000*g* supernatant | 1,200 | 0.62 | 57 | 68,400 | 774 | 0.011 | 100 |
| Phosphocellulose fraction | 150 | 2.1 | 1.94 | 291 | 315 | 1.1 | 42 |
| Crystals | | | | 285 | 285 | 1.0 | 38 |
| With ammonium sulfate fractionation | | | | | | | |
| 37,000*g* supernatant | 950 | 0.84 | 28 | 26,300 | 790 | 0.032 | 100 |
| 45% supernatant | 1,620 | 0.43 | 9 | 14,600 | 690 | 0.048 | 87 |
| 60% precipitate | 203 | 2.80 | 35 | 7,100 | 568 | 0.080 | 73 |
| Phosphocellulose fraction | 58 | 6.30 | 2.92 | 170 | 365 | 2.15 | 46 |

details which differ from those presented for aldolases A and B are presented below.

CRUDE EXTRACT. Frozen rabbit brains (typically 4500 g) were placed in 2.5 volumes of 0.01 M Tris-Cl–0.001 M EDTA–$10^{-4}$ M PMSF[1] (pH 7.5). After partial thawing they were homogenized at low speed for 10 sec in a large commercial blender and then for 30 sec in a Servall Omni-Mixer at 40,000 rpm. The resulting homogenate was centrifuged at 37,500*g* for 1 hr. The precipitate was discarded.

The *ammonium sulfate fractionation* and *phosphocellulose* steps were identical with those presented for aldolase A, except that the phosphocellulose column was washed with one column volume of the sample buffer before the aldolases were eluted with 0.2 M Tris-Cl–0.005 M EDTA–0.0025 M FDP (pH 7.5). The higher Tris-Cl concentration was used to assure simultaneous elution of all of the brain aldolases. A typical elution profile is presented in Figure 3.

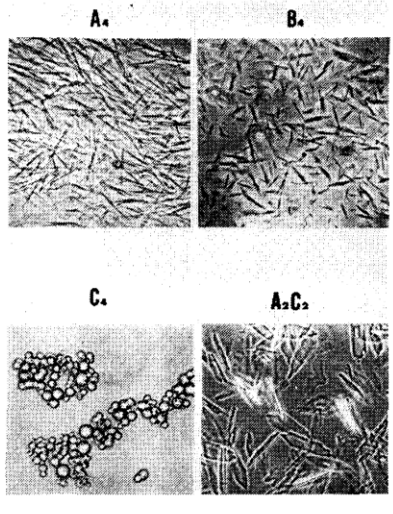


FIGURE 2: Crystalline aldolase preparations. Aldolases crystallized as described in the text were photographed at a magnification of either 1000× (aldolases A and B) or 400× (aldolases C and $A_2C_2$).

DEAE-SEPHADEX CHROMATOGRAPHY. The aldolase-containing fractions from the phosphocellulose column (with specific activities greater than 2) were combined and concentrated to 10–20 mg/ml by ultrafiltration. They were then passed over a Sephadex G-25 column equilibrated with 0.05 M Tris-Cl–0.003 M EDTA–0.2 M sucrose–0.005 M β-mercaptoethanol (pH 8.0). The solution was then applied to a DEAE-Sephadex A-50 column (2.5 × 50 cm) equilibrated with the same buffer and elution was carried out with a linear NaCl gradient (0–0.35 M NaCl in 0.05 M Tris-Cl–0.003 M EDTA–0.20 M sucrose–0.005 M β-mercaptoethanol, pH 8.0) as shown in Figure 4. The members of the A–C hybrid set were resolved from each other by this procedure as shown by the zone electrophoresis (see Figure 5). Each peak was concentrated to approximately 10 mg/ml and precipitated by dialysis against 55% saturated ammonium sulfate–0.001 M EDTA (pH 7.5).

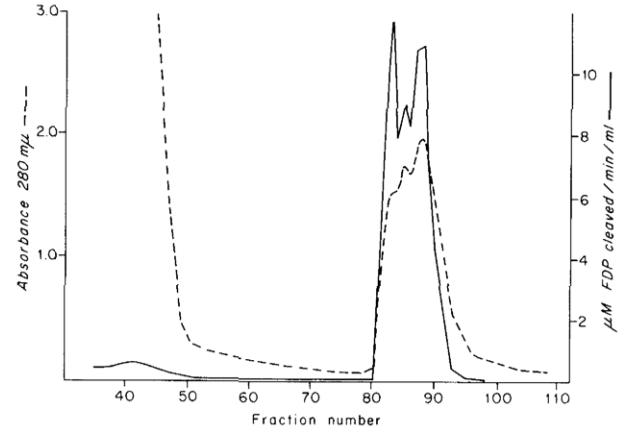


FIGURE 3: Phosphocellulose chromatography of rabbit brain aldolases. The 45–60% ammonium sulfate fraction obtained as described in the text was equilibrated with 0.01 M Tris-Cl–0.001 M EDTA (pH 7.5). The solution was then applied to a 3.7 × 100 cm phosphocellulose column equilibrated with the same buffer. The column was washed with one column volume of the buffer before the aldolases were eluted with 0.2 M Tris-Cl–0.005 M EDTA–0.0025 M FDP (pH 7.5). The volume of the fractions collected was 15 ml.

TABLE III: Purification of the Aldolase A–C Set from Rabbit Brain.

| Fraction | Vol (ml) | Act. (μmoles of FDP/min per ml) | Protein (mg/ml) | Total Protein (mg) | Total Act. | Sp Act. | % Yield |
|---|---|---|---|---|---|---|---|
| 37,000*g* supernatant | 14,000 | 1.78 | 7.5 | 105,000 | 24,900 | 0.24 | 100 |
| 45% $NH_4SO_4$ supernatant | 14,800 | 1.47 | 4.1 | 60,700 | 21,700 | 0.36 | 87 |
| 60% $NH_4SO_4$ precipitate | 1,500 | 11.25 | 26 | 39,000 | 16,900 | 0.43 | 68 |
| Desalted | 2,300 | 8.0 | 17 | 39,000 | 18,400 | 0.47 | 74 |
| Cell $PO_4$ fraction | 403 | 20 | 3.5 | 1,410 | 8,060 | 5.77 | 32 |
| Total DEAE eluted material[a] | | | | | 5,210 | | 21 |

[a] Total activity: $A_4$ = 720, $A_3C$ = 1540, $A_2C_2$ = 1300, $AC_3$ = 980, and $C_4$ = 670.

CRYSTALLIZATION. Crystallization of the middle hybrid, $A_2C_2$, was accomplished either by the method outlined above for aldolase B or by exhaustive dialysis against a 1% glycine solution.

*Purity of the Proteins.* The various aldolase preparations were subjected to cellulose acetate and polyacrylamide disc gel electrophoresis. The results are shown in Figures 5 and 6. A single band of activity was detected for each of the aldolase preparations showing that they were free from contamination by other aldolases.

Protein stains of disc gels of the various aldolases gave an indication of the presence of other contaminating proteins in the preparations. Such electrophoreses of muscle aldolase and liver aldolase B produced single diffuse bands (Figure 6). Most of the members of the A–C hybrid set had only very minor protein contaminants (*e.g.*, the trailing edge of the $C_4$ band and the leading edge of the $A_2C_2$ band). Slight contaminations were more clearly visible in the electrophoreses of $A_3C$. On the other hand, $A_4$ protein from brain was obviously impure. Activity staining of duplicate gels of the brain A preparation showed that the enzyme was the second most rapidly migrating band indicated in Figure 6 by the arrow.

The rather diffuse bands seen for all of the aldolases tested by this technique and noted particularly in preparations of aldolases A and B may be due to the inherent heterodisperse nature of these proteins, a phenomenon which will be elucidated in detail in another publication (M. Kochman and W. J. Rutter, in preparation). Alternatively, the bands may result from modification of the proteins by the oxidizing agents present in the polyacrylamide gels.

The aldolases obtained by the methods described appeared homogeneous in a number of other ways. Sedimentation equilibrium experiments utilizing aldolases A, B, C, and $A_2C_2$ presented in detail in the accompanying publication showed that there was very little molecular weight heterogeneity in any of the proteins tested. Muscle aldolase A, liver aldolase B, and brain aldolase $A_2C_2$ were easily crystallized as shown in Figure 2. Also presented in the same figure are pictures of spherical protein aggregates obtained from attempts to crystallize aldolase C from ammonium sulfate solutions. Several other attempts were made to crystallize aldolase C (*e.g.*, from distilled water or 1% glycine). The enzyme appeared homogeneous by all other criteria applied.

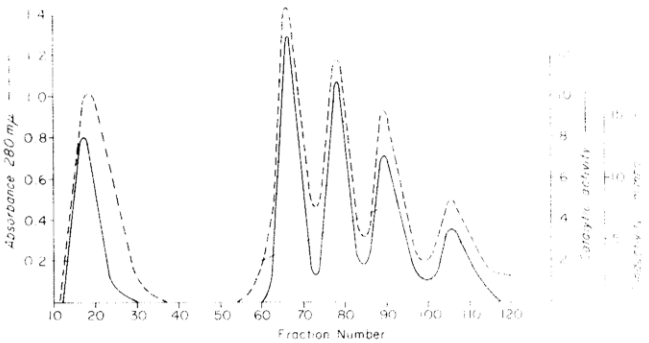


FIGURE 4: DEAE A-50 Sephadex chromatography of the A–C aldolase set isolated from rabbit brain. Aldolases A–C eluted from phosphocellulose were equilibrated with 0.05 M Tris-Cl–0.004 M EDTA–0.2 M sucrose–0.005 M β-mercaptoethanol (pH 8.0). This solution was then applied to a 0.8 × 50 cm DEAE Sephadex A-50 column equilibrated with the same buffer. Elution was carried out with a linear NaCl gradient (0–0.4 M in the same buffer); 2-ml fractions were collected. Aldolase A was present in the breakthrough peak and the order of elution beyond this was $A_3C$, $A_2C_2$, $AC_3$, and $C_4$.

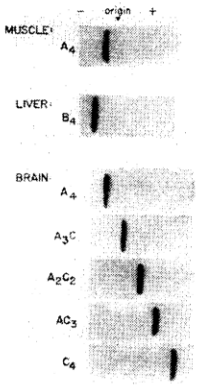


FIGURE 5: Cellulose acetate electrophoresis and activity staining of purified aldolases. Aldolases purified according to the methods described in the text were subjected to electrophoresis on cellulose acetate in 0.06 M sodium barbital (pH 8.6) at 250 V for 90 min. Aldolase activity was detected as previously described (Penhoet *et al.*, 1966).

## Discussion

The methods presented here have evolved from the earlier method of Rajkumar *et al.* (1966) for the isolation of aldolase B. The isolated homomeric aldolases A, B, and C as well as the three A–C hybrids appear to be homogeneous by the criteria employed and can, with the exception of aldolase C, be easily crystallized. The procedures presented here for the isolation of aldolases A and B have several advantages over those previously employed. They are less time consuming and more reproducible than the method of Taylor *et al.* (1948) and Rajkumar *et al.* (1966), respectively, and result in a greater yield of high specific activity enzyme. It is particularly noteworthy that the present procedures do not require the enzymes to stand in crude solutions for long periods of time, thus minimizing the possibility of exopeptidase action.

A number of observations suggest that some degradation or modification of the enzymes may occur in crude extracts. Some variability has been noted in the final specific activities of aldolase B preparations. This may have been the result of peptidase activity in liver extracts or, in addition, there may be some inherent lability of the aldolase B. We have, on occasion, observed the loss of a significant fraction of activity during precipitation of the enzyme with ammonium sulfate or during ethanol fractionation. Such losses have not been observed with other aldolases. Some of our early preparations of aldolase C with low specific activity were shown to yield only two to three carboxyl-terminal tyrosine residues, but four penultimate alanine residues. The addition of $10^{-4}$ PMSF to all buffers prior to the phosphocellulose chromatography seemed to increase the specific activity and also the yield of C-terminal tyrosine residues (Penhoet *et al.*, 1969).

The procedures described herein have been used in our laboratory to prepare aldolases A and B from mouse and rat as well as rabbit tissues, and slight modifications should suffice for the purification of these enzymes from a variety of animal sources.

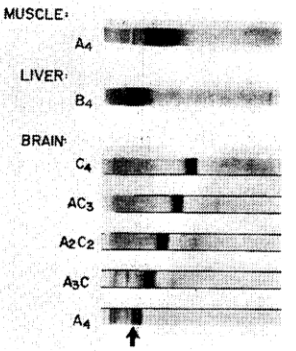


FIGURE 6: Disc gel electrophoresis of purified aldolase proteins. Disc gel electrophoreses were performed according to the methods of Davis (1964) with 5% gels using a pH 8.6 Tris-glycine buffer. The gels were stained with coomassie brilliant blue.

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