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PURIFICATION OF HOMOGENEOUS RAT PHOSPHOFRUCTOKINASE ISOZYMES WITH HIGH SPECIFIC ACTIVITIES

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The purification of rat muscle and liver phosphofructokinase (PFK) isozymes has been greatly facilitated by column chromatographic separation on immobilized Cibacron Blue F_3GA. The homogeneous liver PFK isozyme exhibited a specific activity of greater than 200 units per mg of protein which is nearly two-fold greater than has been previously reported for this isozyme. The yields for this isozyme exceeded 40% of the original activity and the molecular weight of its subunit was about 85,000 as determined by SDS-polyacrylamide gel electrophoresis. The muscle PFK isozyme's specific activity was approximately 265 units/mg of protein which also is about twice the greatest specific activity previously reported. The overall yield for muscle PFK exceeded 50% of the original activity, and the molecular weight of its subunit was approximately 82,000. Using each homogeneous isozyme, antibodies were produced in rabbits; and the immunoglobin-G (IgG) fraction from the sera of these rabbits was highly specific for the PFK isozyme used as an antigen.

Phosphofructokinase (ATP:D-fructose-6-P,1-phosphotransferase, EC 2.7.1.11) is an important glycolytic, regulatory enzyme that catalyzes the rate limiting reaction of glycolysis (1,2). Phosphofructokinase (PFK) is regulated by a number of sophisticated mechanisms. These include modulation by small molecular weight effectors, phosphorylation, and control of its rates of synthesis and/or degradation. Included in this regulatory armamentarium is the distribution of the PFK isozymes in different tissues or organs which apparently complement the physiological role of that tissue or organ. In rat tissues, the predominant PFK isozymes are the forms found in adult liver parenchymal cells and skeletal muscle (3). The existence of a third form in such tissues as the brain, Kupfer cells and platelets is still under active investigation by us (3). The liver PFK isozyme has been purified from liver by a number of investigators (4-7). The specific activities of each preparation vary from 85 to 95 units/mg protein, and the recovery of activity ranges from 15% to 28% of the

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original activity (4-7). The purification methodology for the liver isozyme was adopted from the method of Dunaway and Weber (4). The purification of the muscle enzyme was modified from the procedure of Ling et al. (8). In this paper, the purification of muscle and liver PFK isozymes of high specific activities and excellent yields is reported. Both PFK isozymes are very good antigens and each elicits antibodies that are minimally cross-reactive with the other PFK isozyme.

MATERIALS AND METHODS

Adult male, Wistar rats (200-250 g) were purchased from Harlan/Sprague Dawley, Inc., Indianapolis, IN. All rats were housed in cages with filter caps (Lab Products, Inc., Garfield, NY) and illuminated daily from 7:00 a.m. to 7:00 p.m. Purina Laboratory rat chow was available ad libitum. Following decapitation and exsanguination, livers and skeletal muscle from hind legs were removed.

Cibacron Blue F_3GA gel was purchased from Pierce Chemical Co. (Rockford, IL). Sephacryl S-500 was purchased from Pharmacia Fine Chemicals, and all other reagents were puchased from Sigma Chemical Co. The SDS-polyacrylamide gel electrophoresis was performed as previously described (9). Antisera against muscle or liver PFK isozymes was prepared in rabbits as previously described (4,10). The immunoglobin-G (IgG) fraction was isolated by classical methods (11). PFK catalytic activity was measured using conditions which measure maximal rates of reaction (4). Protein was measured by the method of Bradford using lysozyme as the standard protein (12). This technique for determination of protein was adopted since the components of the column buffer significantly interfere with the method of Lowry et al. (13). If aliquots of purified PFK are dialyzed, the total amounts of protein determined by either method are not significantly different. This indicates that the specific activities reported herein can be compared with the previous work which determined protein levels by the method of Lowry et al. (13).

RESULTS AND DISCUSSION

Purification of the Liver PFK Isozyme. A typical purification for rat liver PFK is shown in Table I. All steps were performed at 4°C except where noted. Livers from thirty rats (350 g) were excised and homogenized in three volumes of extraction buffer, 50 mM Tris-phosphate (pH 8.5), 50 mM NaF, 1 mM ATP, and 10 mM dithiothreitol. The homogenate was centrifuged at 100,000 x g for 1 hr. The resulting supernatant fluid was processed through the 32.5% ammonium sulfate step as previously described (4). The precipitate from the previous step was resuspended in a minimum volume column buffer (50 mM Tris-phosphate, pH 8.0, 50 mM NaF, 0.1 mM Na_2EDTA, 0.05 mM fructose-1,P_2, and 10 mM dithiothreitol). Then the resulting opaque solution was introduced onto a column (2.6 x 40) which had been packed with Cibacron Blue F_3GA gel

TABLE I. Purification of Rat Liver Phosphofructokinase

Step	Total Activity (Units)	Total Protein (mg)	Specific Activity (Units/mg protein)	Yield (%)
Supernatant Fluid	1,850	40,300	.046	100
Heat Step	1,750	10,100	.173	94
Ammonium Sulfate Precipitate	1,670	1,080	1.55	90
Cibacron Blue F_3GA Step	1,090	12.1	90.1	65
Sephacryl S-500 Leading Edge	556	2.7	206	30
Sephacryl S-500 Trailing Edge	370	2.1	175	20

and which had been equilibrated with column buffer. After loading, the column was washed until the absorbance at 280 nm returned to baseline. Following this, the column was washed with column buffer plus 0.15 mM ADP. This step was suggested by the work of Kagimoto and Uyeda (6).

PFK activity was eluted from this column in a large final volume (100 ml) using column buffer plus 2 mM ATP and 2 mM fructose-6-P. Fractions of greatest activity were pooled and precipitated with ammonium sulfate (0.31 g/ml). The resulting precipitate was resuspended in a minimum volume of column buffer. As can be seen from Table I about 65% of the original activity was recovered with a specific activity of approximately 90 units/mg protein. SDS-polyacrylamide gel electrophoresis of this preparation indicated 5 to 6 minor bands and a major band which corresponds to a molecular weight of 85,000 and which is presumably the liver PFK subunit. The pooled fractions from this step were chromatographed on a Sephacryl S-500 column (1.6 x 70 cm) which had been equilibrated with column buffer. Under these conditions the majority of the PFK activity was eluted immediately after the void volume. The fractions comprising the leading edge of the activity peak and the peak fractions were combined. The activity in these fractions was precipitated with ammonium sulfate as described earlier, and stored in liquid nitrogen in the following

buffer, 20 mM potassium phosphate (pH 7.6), 3 mM $MgSO_4$, 1 mM fructose-6-P, 0.1 mM EDTA, 10 mM dithiothreitol, and 20% (w/v) glycerol, which was originally described by Reinhart and Lardy (7). Between 55% and 60% of the recovered PFK activity was found in the leading edge fraction. The recovery of activity in this fraction was approximately 30% of the original activity and can be improved to nearly 45% by rechromatography of the trailing edge combined fractions. We preferred to combine trailing edge fractions from several purification attempts and then rechromatograph on Sephacryl S-500 as described above. Theoretically, repeated chromatography on this gel should allow recoveries exceeding 45%. The specific activity of the liver PFK in the leading edge fraction was 206 units/mg protein (Table I) which is greater than has been reported by other investigators (4-7). The pooled leading edge factions were submitted to SDS-polyacrylamide gel electrophoresis, and the results are shown in the inset of Fig. 1. It is apparent that this enzyme preparation was homogeneous and that the liver enzyme was composed of a single subunit based on molecular weight. Relative to the standards which were employed, the subunit molecular weight of the liver PFK subunit was estimated to be 85,000 (Fig. 1).

Purification of the Muscle PFK Isozyme.

The PFK isozyme from adult muscle was purified by modifying the procedure of Ling et al. (8). After chromatography on DEAE-cellulose, the samples of highest activity were pooled, precipitated with ammonium sulfate, and resuspended in column buffer as described for the rat liver isozyme. This solution was added directly on a Cibacron Blue F_3GA gel column and processed as described for the liver isozyme. The specific activities and yields of the prior steps were in good agreement with the steps in common with those of Ling et al. (8). As can be seen in Table II, this technique permitted a good recovery of PFK activity (52%) and resulted in a enzyme of high specific activity (265 units/mg protein). It is apparent from the inset of Fig. 1 that this purification procedure produces homogeneous muscle PFK. Further, it was shown in this figure that the subunit molecular weight of the muscle PFK isozyme was 82,000. For this enzyme, Ling et al. reported a specific activity of 130 unit/mg pro-

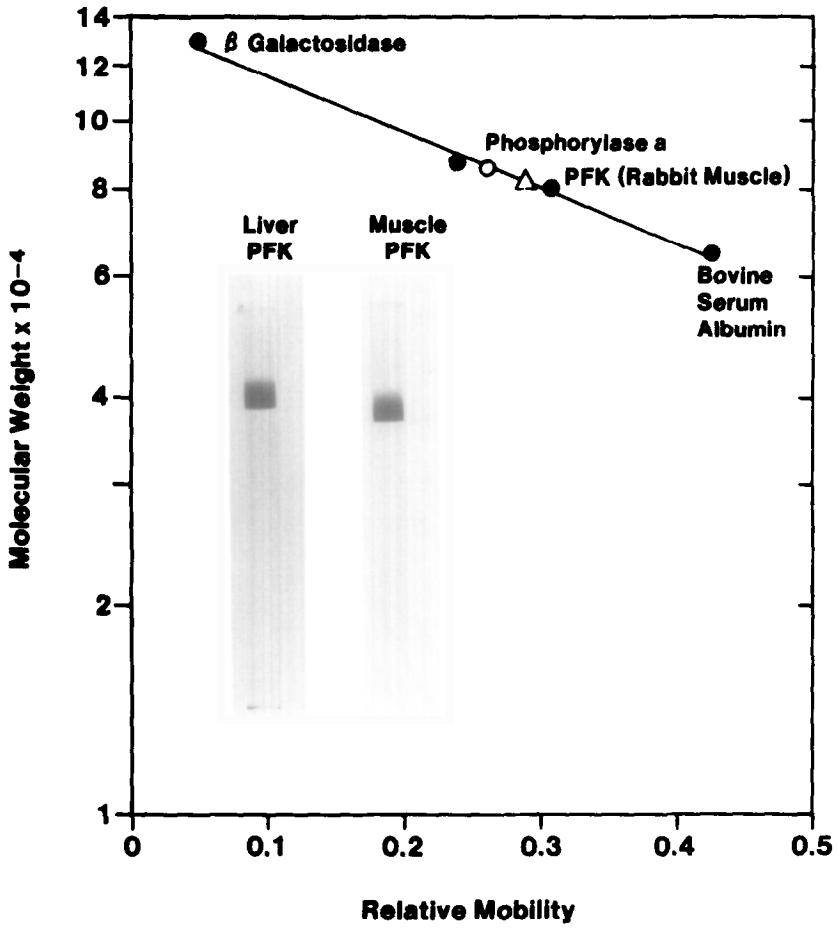

Figure 1. Determination of the Subunit Molecular Weights of Muscle and Liver PFK Isozymes by SDS-polyacrylamide Electrophoresis. Approximately 150 µg of protein was added to each 7.5% gel and developed. For more information see reference 9. Open circle presents the liver PFK isozyme subunit; and the open triangle represents is the muscle PFK subunit. The insets are the tube gels of the SDS-treated muscle and PFK isozymes.

tein; and Uyeda et al., using their technique, demonstrated a specific activity of 120 units/mg protein (8,14). Odeide et al. and Tanaka et al., using different protocols, found specific activities of 125 and 150 units/mg protein, respectively (15,16). Thus, our technique yielded an enzyme with about twice the specific activity reported by other investigators for rat muscle PFK.

Immunological Studies.

Antisera to each isozyme was separately induced in rabbits using each of the purified PFK isozymes. Titration of the purified muscle and liver PFK isozymes with the different anti-IgG fractions is shown in Fig. 2A. Each IgG

TABLE II. Purification of Rat Muscle Phosphofructokinase

Step	Total Activity (Units)	Total Protein (mg)	Specific Activity (Units/mg protein)	Yield (%)
Supernatant Fluid	10,080	6,520	1.55	100
Alcohol Step	8,184	99	82.7	81
DEAE Step	7,350	49	150	73
Cibacron Blue F_3GA Step	5,250	19.8	265	52

129 g of skeletal leg muscle

fraction completely precipitated the corresponding PFK isozyme (or original antigen) at low concentrations of antisera. Also, very little cross-reactivity was noted. As can be seen in Figure 2B, PFK activity in muscle supernatant fluid was only slightly affected by liver PFK anti-IgG and nearly completely inactivated by 20 µl of muscle PFK anti-IgG. Using liver supernatant fluid,

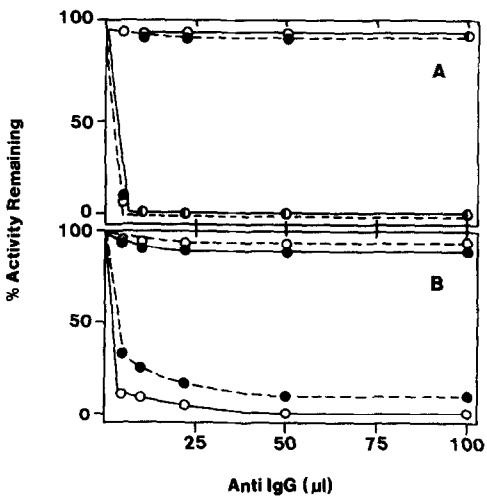

Figure 2. Immunological Titration with the Muscle PFK and Liver PFK Anti-IgG Fractions.
Panel A represents the results of titration of purified liver PFK (●) and muscle PFK (○) with liver PFK anti-IgG fraction (dashed line) or muscle PFK anti-IgG fraction (solid line). Panel B represents the data obtained from titration of liver (●) and muscle (○) supernatant fluid with liver PFK anti-IgG fraction (dashed line) or muscle PFK anti-IgG fraction (solid line). Each supernatant fluid (100 µl) was incubated at room temperature for 1.5 hrs with the indicated volume of each anti-IgG fraction after attaining a final volume of 350 µl with different aliquots of phosphate buffered saline.

the PFK activity was inhibited by about 10% with muscle PFK anti-IgG and about 90% by liver PFK anti-IgG. Since the liver has been shown to have two PFK isozymes, it was not surprising that liver supernate PFK activity was sensitive to both antisera (4). Earlier work had shown by separation via DEAE-cellulose column chromatography that the minor PFK isozyme was about 15% of the total liver PFK activity and was found in Kupfer cells (4,17). Also, the major PFK isozyme, which is 85% of the total liver PFK activity, was located in the hepatic parenchymal cells (4,17). Titration of a crude preparation of the minor isozyme with either antiserum indicates that it was about 50% neutralized with liver PFK anti-IgG and 85% inhibited by muscle PFK anti-IgG (3). Further, as seen in Fig. 2A, the liver PFK anti-IgG completely neutralized the major liver PFK isozyme and hardly affected the muscle PFK isozyme. Thus, the sensitivity of the PFK activity in liver supernate to muscle PFK anti-IgG and incomplete neutralization by liver PFK anti-IgG were due to the presence of the minor liver PFK isozyme. These results indicate that cross-reactivity of the anti-IgG fractions is minimal and that these anti-IgG fractions are useful tools for the detection of liver or muscle PFK antigenic determinants. Earlier work by our group using antisera prepared with less pure PFK isozymes has shown that muscle and liver PFK isozymes are antigenically different (4,18). However, the work reported herein confirmed and extended that observation by examing the effects of the two anti-IgG fractions on the minor liver PFK isozyme, as well as the homogeneous muscle and major liver PFK isozymes which exhibit high specific activities.

In this report, we have described a simple and fast method for the preparation of homogeneous PFK isozymes from rat muscle and liver. Each isozyme can be obtained in excellent yields which approach 50% recovery of the original activities and with high specific activites of about 200 units/mg protein for the liver isozyme and 265 units/mg protein for the muscle isozyme. The molecular weights of the liver and muscle PFK subunits were 85,000 and 82,000, respectively. Apparently, the muscle PFK subunit has a smaller molecular weight than the liver subunit. Good agreement with previous reports for sub-

unit molecular weights of the rat PFK isozymes is found (5,7,9). Also, antibodies directed against each of these PFK isozymes appear to be highly specific for the isozyme which served as the antigen. Therefore, these preparations of liver parenchymal cell and skeletal muscle PFK isozymes and the antibodies that they elicit will be assets in many biochemical and physiological studies, such as protein turnover studies and examination of the effect of phosphorylation on the catalytic activities of each PFK isozyme.

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REFERENCES

1. Uyeda, K. (1979) Adv. Enzymol. Relat. Areas Mol. Biol. 48, 193-244.
2. Hoffmann, E. (1976) Reviews of Physiology, Biochemistry and Pharmacology 75, 1-68.
3. Dunaway, G.A. (1983) Mole. and Cell. Biochem. in press.
4. Dunaway, G.A. and Weber, G. (1974) Arch. Biochem. Biophys. 162, 620-628.
5. Brand, I.A. and Söling, H.-D. (1974) J. Biol. Chem. 249, 7824-7831.
6. Kagimoto, T. and Uyeda, K. (1979) J. Biol. Chem. 254, 5584-5587.
7. Reinhart, G.D. and Lardy, H.A. (1980) Biochem. 19, 1477-1484.
8. Ling, K.-H., Marcus, F. and Lardy, H.A. (1965) J. Biol. Chem. 240, 1893-1899.
9. Dunaway, G.A., Leung, G.L.-Y., Thrasher, J.R. and Cooper, M.D. (1978) J. Biol. Chem. 253, 7460-7463.
10. Thrasher, J.R., Cooper, M.D. and Dunaway, G.A. (1981) J. Biol. Chem. 256, 7844-7848.
11. Garvey, J.S., Cremer, M.E. and Sussdorf, D.H. (1977) in Methods in Immunology 3rd Ed., pp. 218-226, Addison Wesley Publishing Co. Reading, MA.
12. Bradford, M. (1976) Anal. Biochem. 72, 248-254.
13. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randal, R.J. (1951) J. Biol. Chem. 193, 265-275.
14. Uyeda, K., Miyatake, A., Luby, L.J. and Richards, E.G. (1978) J. Biol. Chem. 253, 8319-8327.
15. Odeide, R., Guilloton, M., Dupuis, B., Ravon, D. and Rosenberg, A.J. (1968) Bull. Soc. Chim. Biol. 50, 2023-2033.
16. Tanaka, T., An, T. and Sakoue, Y. (1971) J. Biochem. 69, 609-612.
17. Dunaway, G.A., Leung, G.L.-Y., Cooper, M.D., Thrasher, J.R. and Wagle, S.R. (1978) Biochem. Biophys. Res. Commun. 80, 71-79.
18. Dunaway, G.A., Naqui, D., Kruep, D., Thrasher, J.R. and Morris, H.P. (1981) Arch. Biochem. Biophys. 212, 1-8.