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PURIFICATION OF HUMAN LEUCOCYTE PYRUVATE KINASE

D. KECHEMIR, I. MAX-AUDIT, M.C. CALVIN-PREVAL and R. ROSA*

INSERM U. 91, Hôpital Henri Mondor, Créteil 94010 (France)

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SUMMARY

The M_2 form of pyruvate kinase (M_2-PK) was purified from human leucocytes by a new method involving a succession of two different Dyematrex agarose chromatographies. The main step consisted of an orange dye affinity column with elution by fructose-1,6-diphosphate. This purification procedure allowed us to obtain M_2-PK with a specific activity of 433 I.U./mg of protein, i.e. a 188-fold purification with an overall yield of 33%. The homogeneity of this preparation was verified by sodium dodecyl sulphate polyacrylamide gel electrophoresis and double immunodiffusion in Ouchterlony plates. Anti-M_2-PK antibodies obtained from rabbit neutralized the enzyme activity. Their specificity with regard to other types of PK showed that anti-M_2-PK also reacted with M_1-PK but not with R-PK.

INTRODUCTION

Pyruvate kinase (ATP: pyruvate phosphotransferase, EC 2.7.1.40, PK) is an important regulatory glycolytic enzyme which converts phosphoenol pyruvate into pyruvate and concomitantly ADP into ATP. Four different isozymic forms of pyruvate kinase (L, R, M_1 and M_2) are present in human tissues. Their expression differs from one tissue to another [1]. These isozymic forms exhibit differences in electrophoretic, kinetic and immunological properties. L-Type PK (L-PK) specific of the liver and R-PK specific of the red blood cell (RBC) are encoded by the same gene but their mRNAs differ [2]. Two other types of PK, the M_1-PK located in skeletal muscle and the M_2-PK widely distributed in most mammalian tissues [3], display immunological cross-reactivity. The M_2-PK is considered to be the prototype PK, whereas the other isozymes are regarded as differentiated forms [1, 3]. Human M_2-PK has previously been purified from human lung and kidney [4, 5] by classical ion-exchange chromatographic methods; Harkins et al. [5] included a dye ligand

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chromatography step, but the overall yield was low. Conversely, human R-PK and L-PK have already been purified by various methods, including dye ligand chromatography [6, 7]. Our aims were to develop a rapid and simple method for the purification of M_2-PK from leucocytes by using dye ligand chromatography, and to obtain anti-M_2-PK antibodies.

EXPERIMENTAL

All substrates and commercial enzymes were purchased from Boehringer Mannheim (F.R.G.) unless otherwise specified; α-cellulose Sigma Type 50 and NADH were products of Sigma (St. Louis, MO, U.S.A.). Acrylamide, bis-acrylamide and diisopropyl fluorophosphate (DIFP) were supplied by Fluka (Buchs, Switzerland). Dyematrex gel blue A and orange were from Amicon (Lexington, MA, U.S.A.). Freund's complete and incomplete adjuvants were obtained from Gibco (Grand Island, NY, U.S.A.). Buffer salts were from Merck (Darmstadt, F.R.G.). Cellogel strips were purchased from Medical Products, Chemetron (Milan, Italy). Nitrocellulose (NC) membranes (pore size 0.45 μm) were purchased from Sartorius (Göttingen, F.R.G.) and affinity-purified goat anti-rabbit immunoglobulin G (IgG) (H + L) horseradish peroxidase conjugate from Bio-Rad (Richmond, CA, U.S.A.).

PK was assayed by the classical method using the reaction coupled with lactate dehydrogenase (LDH) activity, as previously described [8]. Glyceraldehyde phosphate dehydrogenase (GAPDH) and LDH were assayed by the method recommended by the international committee for standardization in haematology [9]. Protein concentrations were measured according to the method of Lowry et al. [10], with bovine serum albumin as the working standard. Samples were concentrated in ultrafiltration cells obtained from Amicon (Lexington, MA, U.S.A.).

Electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli [11]. The proteins were stained with Coomassie blue.

Cellogel electrophoresis was performed in 0.075 *M* Tris maleic acid sodium hydroxide buffer (pH 5.5) at 4°C for 3 h at 180 V, as previously described [12]. After completion of electrophoresis, one strip was stained for PK activity. The second strip was used for immunoblotting on NC according to the method developed for bisphosphoglyceromutase [13]. Then immunodetection of PK was performed on the NC using specific anti-M_2-PK antibodies coupled to anti-rabbit IgG horseradish peroxidase conjugate.

Immunological studies

The anti-M_2-PK antiserum was obtained from rabbit. The animal received 1.25 mg of a completely purified enzyme by intramuscular injection. The first injection contained 0.25 mg of purified M_2-PK emulsified with complete Freund's adjuvant, and the four subsequent injections each contained 0.25 mg of enzyme emulsified with incomplete Freund's adjuvant. The first three injections were made every fifteen days. After a three-month gap, two injec-

tions were made one week apart. A further week later, the rabbit was killed and the blood collected. The serum was heated for 30 min at 56°C and stored frozen.

The anti-M_2-PK antiserum was tested by double-immunodiffusion technique according to the method of Ouchterlony [14]. Immunoneutralization was performed according to a method previously described for R-PK [12]. The samples were mixed with increasing concentrations of anti-M_2-PK antiserum in 100 m*M* Tris—HCl buffer (pH 8.0) containing 100 m*M* potassium chloride, 0.1 m*M* fructose-1,6-diphosphate (FDP), 1 m*M* EDTA, 1 m*M* ϵ-amino-*n*-caproic acid, 1 m*M* DIFP, 0.1 m*M* dithiothreitol, 2 mg/ml bovine albumin and 500 m*M* sucrose. After incubation for 1 h at 37°C and overnight at 4°C, the tubes were centrifuged at 20 000 *g* and the residual PK activity was assayed in the supernatant.

Purification procedures

The complete purification procedure is summarized in Table I and the different steps are described below.

Isolation of leucocytes and preparation of the crude extract. Enriched leucocyte preparations were obtained by leucopheresis. Contaminating RBCs were eliminated by selective lysis as described by Kahn et al. [15]. The leucocytes washed with cold 0.9% sodium chloride containing 2 m*M* ϵ-amino-*n*-caproic acid and 2 m*M* EDTA, were lysed with a homogenizer in two volumes of ice-cold buffer A (see following step). Cold toluene was added up to a 10% (v/v) concentration and vigorously emulsified with the extract. After centrifugation for 60 min at 20 000 *g*, the upper fatty layer was drawn off and the clear supernatant was used for the purification of PK.

All the steps of the purification procedure were done at 4°C. Unless otherwise indicated, all the buffers contained 0.4 m*M* EDTA, 2 m*M* ϵ-amino-*n*-caproic acid and 0.5 m*M* dithiothreitol.

Dyematrex gel blue A column chromatography. A 9-ml volume of a crude extract was applied on a Dyematrex gel column (3.5 × 1.5 cm) previously equilibrated with buffer A (10 m*M* potassium phosphate at pH 7.0 containing 5 m*M* magnesium sulphate and 0.1 m*M* FDP). The flow-rate was 100 ml/h.

TABLE I

PURIFICATION OF LEUCOCYTE PYRUVATE KINASE

Purification step	Total protein (mg)	Total activity (I.U.)	Specific activity I.U./mg of protein	Purification (fold)	Recovery (%)
Crude extract	169	393	2.3	1	100
Dyematrex blue A chromatography	22	360	17	7	91
50—75% Ammonium sulphate precipitation	8	164	20	9	42
Dyematrex orange chromatography	0.3	130	433	188	33

A protein peak that did not contain PK activity was eluted with 200 ml of buffer A. Then 150 ml of a potassium chloride gradient (0 to 500 m*M*) were applied to the column, and M_2-PK began to be eluted at ca. 66 m*M* potassium chloride. Fractions with high PK activity were pooled.

Ammonium sulphate fractionation. The PK-containing eluate from the Dyematrex blue A column was brought to 50% ammonium sulphate saturation by adding solid crystals. The pH of the solution was maintained at 7.0 by dropwise addition of 1 *M* sodium hydroxide. The solution was left for 30 min, then centrifuged at 27 000 *g* for 30 min. The pellet was discarded. The supernatant was brought to 75% ammonium sulphate saturation by adding solid crystals and left for 30 min before centrifugation at 27 000 *g* for 30 min. Then the pellet was dissolved in a small amount of buffer B (see following step). The M_2-PK isozyme can be stored for at least six weeks at −80°C as 75% saturated ammonium sulphate suspension without any significant loss of activity.

Dyematrex gel orange column chromatography. The Dyematrex orange

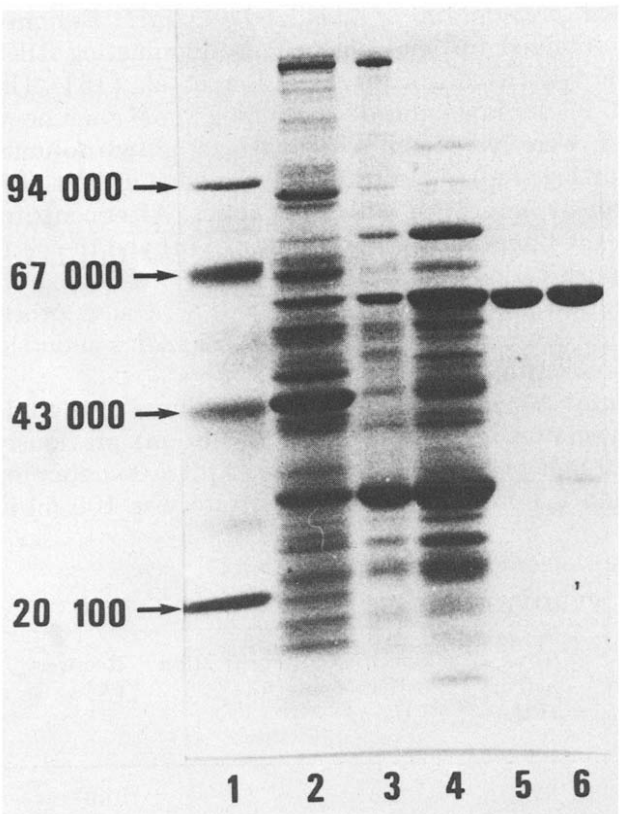

Fig. 1. SDS-PAGE of M_2-PK from leucocytes at different stages of purification. (1) Mixture of purified proteins of known molecular mass: phosphorylase b (94 000), bovine serum albumin (67 000), ovalbumine (43 000), soybean trypsin inhibitor (20 100); (2) leucocyte crude extract (100 μg); (3) Dyematrex blue A column eluate (23 μg); (4) 75% ammonium sulphate precipitate (57 μg); (5) Dyematrex orange column eluate (5 μg); (6) Dyematrex orange column eluate in absence of a wash with NADH (6 μg).

column (3 × 1 cm) was equilibrated with 25 m*M* Tris—HCl buffer (pH 7.0) containing 5 m*M* magnesium chloride (buffer B). The flow-rate was 25 ml/h. The ammonium sulphate precipitate from the previous step was dialysed against buffer B and applied to the orange column, allowed to penetrate and left there overnight. It was necessary to leave the enzyme in contact with the resin for such a long time to minimize the outcome of PK with buffer B. The following day, the column was washed with buffer B until no more protein was eluted. After the column had been washed with buffer C (identical with buffer B except for its containing 25 m*M* potassium chloride and the pH being 8.5), a second protein peak showing less than 10% activity was eliminated. Then 0.25 m*M* NADH was added to buffer C to promote the elution of the last

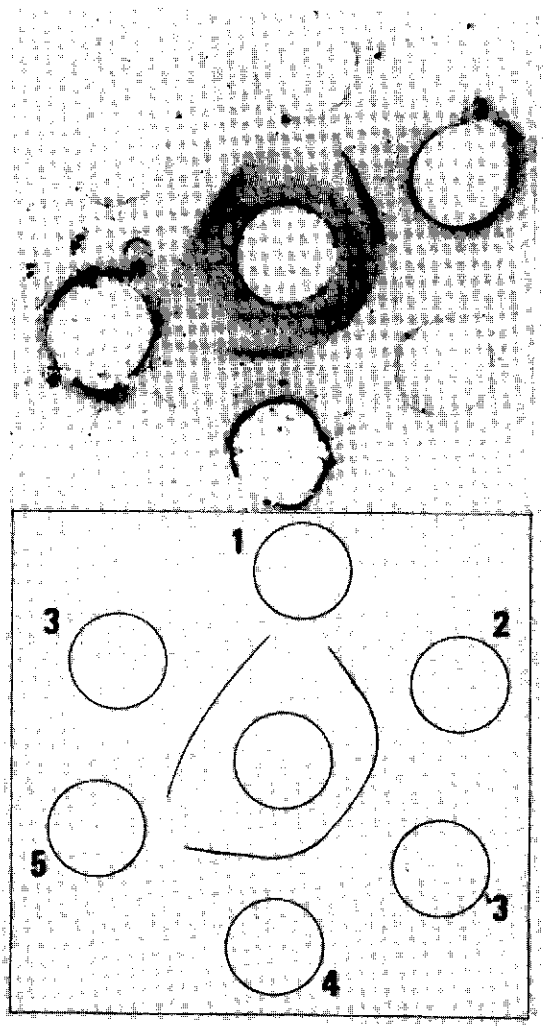

Fig. 2. Double-immunodiffusion analysis of antiserum. (Centre well) 20 μl of rabbit anti-M_2-PK antiserum; (1) 20 μl of human haemolysate (R-PK) (5 mg); (2) 20 μl of human leucocyte extract (0.2 mg); (3) 20 μl of purified M_2-PK (4 μg); (4) 20 μl of human muscle extract (M_1-PK) (0.2 mg); (5) 20 μl of purified R-PK (10 μg).

contaminants, GAPDH and LDH (see Fig. 1, lane 6). The M_2-PK was eluted with buffer C containing 0.5 m*M* FDP. Tubes containing the highest enzymatic activity were pooled. The M_2-PK was highly purified (Fig. 1). The eluted PK was concentrated in an ultrafiltration cell to a final volume of 1 ml, and sucrose was added to a final concentration of 500 m*M* to protect PK activity.

We first tried to elute M_2-PK with FDP just after washing the column with buffer C, but SDS-PAGE showed that the M_2-PK eluate contained contaminants (Fig. 1, lane 6). A search of the enzymatic activities of proteins of molecular weight approximately similar to these contaminants revealed that LDH and GAPDH were responsible. Thus we used a common substrate NADH to promote their elution before the specific elution of M_2-PK by FDP.

RESULTS

The purity of the enzyme was tested by SDS-PAGE. Fig. 1 illustrates the various purification steps and shows that the main step is Dyematrex orange chromatography (lane 5). This also appears in Table I: after the first two steps, the enzyme was purified nine-fold while the Dyematrex orange chromatography allowed an increase in specific activity of more than twenty-fold in one step. The final result of the purification procedure is an enzyme purified 188-fold with an overall yield of 33% and a specific activity of 433 I.U./mg of protein.

Immunological studies

The results of immunodiffusion studies using rabbit anti-human M_2-PK

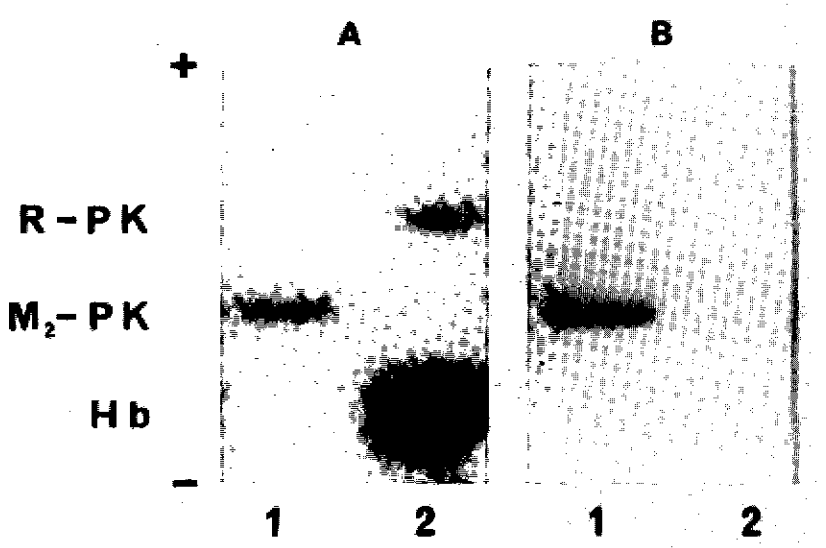

Fig. 3. Immunodetection of M_2-PK after cellogel electrophoresis. (1) 5 μl of crude leucocyte extract (1 I.U./ml); (2) 5 μl of RBC lysate (0.5 I.U./ml). (A) Activity staining: PK activity was detected under UV light with 1.5 m*M* ADP, 1.5 m*M* PEP, 0.12 m*M* NADH and 5 I.U. LDH; (B) immunodetection was performed according to Calvin et al. [13] with the following modification: 1-h incubation with a 400-fold diluted specific anti-M_2-PK antiserum was used.

antiserum are shown in Fig. 2. A single precipitation line was seen with the pure enzyme, as with the crude leucocyte extract and with a human muscle extract containing M_1-PK. No precipitation line was seen with the R-PK from human RBCs.

Immunodetection after cellogel electrophoresis is shown in Fig. 3. The M_2-PK electrophoresis pattern of a crude extract is the same as those obtained by immunodetection or enzymatic staining: a single band migrating at the same level is obtained by these two different methods. Conversely, R-PK from RBCs appears by enzymatic staining but not by immunodetection with the anti M_2-PK antiserum.

Immunoneutralization. As can be seen in Fig. 4, M_2-PK was almost completely neutralized by anti-M_2-PK antiserum, and under our conditions 50% inhibition was obtained with ca. 1.5 μl of antiserum. On the contrary, the anti-M_2-PK antiserum did not neutralize R-PK activity. By extrapolating the linear portion of the curve of neutralization, a titre of 5.3 I.U. of neutralized M_2-PK per millilitre of antiserum was obtained.

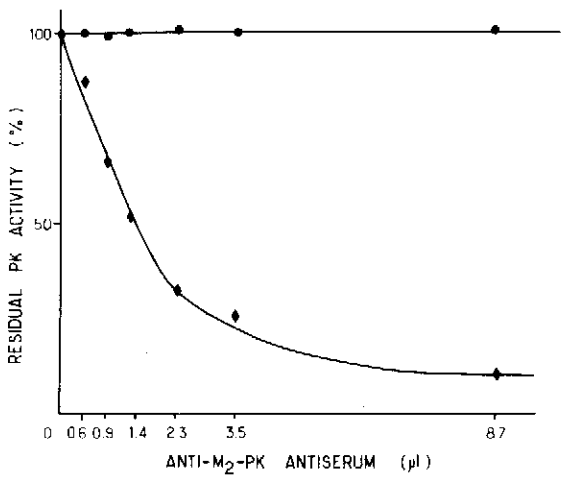

Fig. 4. Immunoneutralization of M_2-PK from purified preparation. Progressive volumes of antiserum were added in a total volume of 70 μl. (♦) Purified M_2-PK; (•) R-PK from human RBC. The activity is expressed as a percentage of the original activity. No inhibition of PK activity occurred with preimmune rabbit serum (not shown).

DISCUSSION

Purification of human M_2-PK is difficult because this enzyme is unstable. Thus it is important to use a rapid procedure to purify this enzyme. In this paper we have reported a new chromatographic procedure based on successive columns of Dyematrex gels. The method, which involves three steps, has several advantages. It is fast and easy to handle, and the columns have a very small volume and can be used several times (at least five) with a good reproducibility. The 33% yield we have obtained is good for human M_2-PK compared with those of Harkins et al. [5] and Corcoran et al. [4], who obtained 3.7% in kidney and 4% in lung, respectively. Our better yield can probably be

explained by the rapidity and the small number of steps. We selected Dyematrex blue A for the first step because it binds M_2-PK tightly and because it binds a large amount of protein compared with Dyematrex orange (see Table I). This allowed us to process a large sample. The ammonium sulphate fractionation had several purposes: (1) elimination of FDP used as a protector in the first step but as a specific ligand for elution of M_2-PK from the next column; (2) elimination of an eventual contamination by R-PK [16] from red cells remaining with the leucocytes; (3) concentration of the eluate. The determinant step of the procedure is the orange dye chromatography, which requires FDP to elute M_2-PK from the column. FDP is an allosteric activator of M_2-PK [5]. This activator breaks the link between the orange dye and M_2-PK, probably by promoting a conformational change of M_2-PK, as previously observed by Marie and Kahn [6] for R-PK purification. The purity of the enzyme was checked by SDS-PAGE (Fig. 1) and double immunodiffusion (Fig. 2). The antibodies obtained cross-react with the M_1-PK from human muscle but not at all with R-PK from RBCs by any method we used: double immunodiffusion, immunoneutralization and immunodetection after cellogel electrophoresis. Similar results were obtained by Harkins et al. [5] in human and by Imamura et al. [3] in rat.

In conclusion, the described method of purification of human leucocyte PK allows us to obtain in a short time a purified M_2-PK with a yield of 33% (a good yield for this enzyme). With this purified M_2-PK, antibodies were raised in rabbit, with a titre high enough to allow high sensitivity immunodetection and immunoneutralization.

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