PURIFICATION OF HUMAN ERYTHROCYTE PHOSPHOFRUCTOKINASE

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1. Introduction

Phosphofructokinase* has been isolated from several mammalian tissues in highly purified or even crystallized forms [1–6]. Human PFK received greater attention when a new human disease was detected, which could be characterized by a deficiency of muscle PFK and a decrease of the enzyme in red blood cells to about 50% of normal. These findings led to comparative studies of various properties of both enzymes, which revealed characteristic differences between them [7–9]. In these studies only partially purified erythrocyte PFK with a specific activity of 12 was used [7].

Recently, we described the occurrence of various oligomeric forms of human erythrocyte PFK, and determined the molecular weights of their SDS cleavage products [10].

In this paper the procedure for getting a highly purified, stable and homogeneous human erythrocyte PFK preparation with a specific activity of about 130 is described. Some special properties of the enzyme are also presented.

2. Materials and methods

PFK activity was measured in the coupled optical test at 25° [10]. Enzyme activity is given in International Units (IU), defined as μmoles FDP formed per min in the standard assay.

Protein concentrations were determined according to [11] with human serum albumin as a standard or spectrophotometrically at 280 nm [12]. Hemoglobin was measured by the cyanmethemoglobin method [13].

* *Abbreviations*:
F6P, fructose 6-phosphate; FDP, fructose-1,6-diphosphate; PFK, phosphofructokinase; SDS, sodium dodecylsulphate.

3. Results

3.1. *Purification procedure*

The following method describes the preparation of PFK from human red blood cells obtained from a blood bank. At least the first steps of the method are similar to the procedure of Layzer et al. [7]. However, the ionic composition and pH of various media were changed as a result of numerous experiments to improve enzyme stability and efficiency of the preparation procedure. In our experience the yield, purity and stability of the final preparation are dependent on the first purification steps, thus these are therefore described in detail. All operations were carried out at 0–2° unless otherwise indicated. The blood was centrifuged and the erythrocytes were washed 3 times with 0.9% NaCl. In general, all potassium phosphate buffers used in the subsequent steps contained 10 mM ammonium sulphate, 1 mM EDTA, 0.5 mM FDP, 0.1 mM ATP and 5 mM mercaptoethanol.

3.1.1. Step 1: Hemolysis

The washed erythrocytes were mixed with an equal volume of 10 mM phosphate buffer (pH 8.0) and stirred for one hr. After that the stroma was removed by centrifugation (15,000 *g*, 30 min).

3.1.2. Step 2: Adsorption on DEAE-cellulose

DEAE-cellulose (50 g/l hemolysate) equilibrated with 10 mM phosphate buffer (pH 8.0) was poured into the stroma-free hemolysate and stirred for an

Table 1
Purification of human erythrocyte phosphofructokinase.

Step	Volume (ml)	Total activity (IU)	Total protein (mg)	Specific activity (IU/mg protein)	Purification	Yield (%)
1. Hemolysate	4800	1920	585,000	0.003*	1	100
2. Batch DEAE-cellulose	1410	1560	5,250	0.3	100	81
3. Ammonium sulphate fractionation	240	1460	1,620	0.9	300	76
4. Heat treatment	230	1310	350	3.7	1,230	68
5. Sephadex G-200	3.5	1080	54	20	6,700	56
6. Sepharose 4 B	1.5	970	7.1	136	44,000	51

* The specific activity of the enzyme in the hemolysate is referred to hemoglobin.

additional hr. Thereafter the DEAE-cellulose was separated by centrifugation and washed several times with 10 mM and 50 mM phosphate buffer to remove hemoglobin. The enzyme was eluted from DEAE-cellulose with 250 mM phosphate buffer.

3.1.3. Step 3: Ammonium sulphate fractionation

Solid ammonium sulphate was slowly added to the eluate from the DEAE-cellulose to a final saturation of 0.45 adjusting the pH to 8.0 with 1 M ammonia. The suspension was stirred for 30 min and after centrifugation (26,000 *g*, 30 min) the precipitate was dissolved in 100 mM phosphate buffer to a volume of about one tenth of that of the erythrocyte sediment. To this solution, solid ammonium sulphate was again added to a final saturation of 0.25. After 30 min the precipitate was removed by centrifugation and discarded.

3.1.4. Step 4: Heat treatment

The supernatant was rapidly heated with stirring in a jacketted vessel to 65° and kept at this temperature for 30 min. After rapidly cooling to 0°, the suspension was centrifuged at 26,000 *g*, the precipitate discarded, and ammonium sulphate added to the supernatant to 0.45 saturation. After 30 min, the precipitate was col-

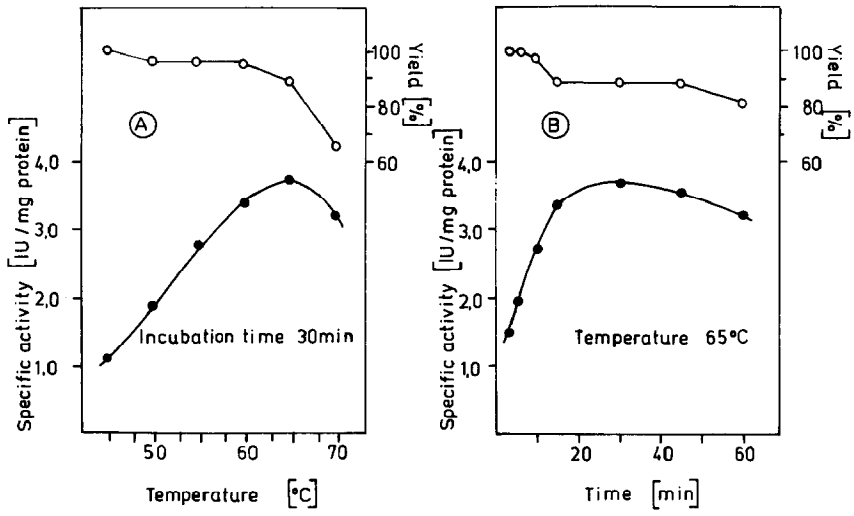

Fig. 1. Stability of erythrocyte phosphofructokinase as functions of temperature and time. (A) Dependence on temperature, incubation time 30 min, and (B) dependence on time at 65°. ●—● specific activity, ○—○ enzyme yield.

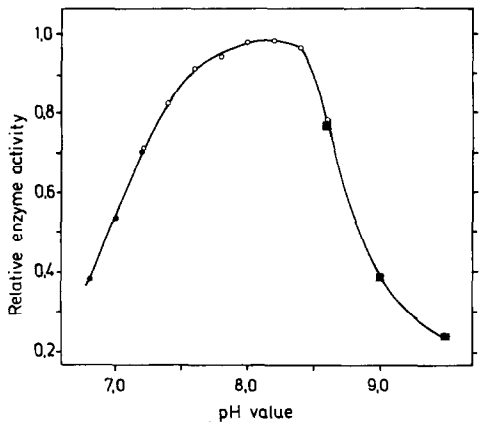

Fig. 2. pH-dependence of erythrocyte phosphofructokinase activity. ●—● imidazole/HCl buffer, ○—○ Tris/HCl buffer, ■—■ Veronal-Na buffer.

lected by centrifugation at 26,000 *g* and redissolved in a small volume of 100 mM phosphate buffer. Insoluble residues were removed by centrifugation.

3.1.5. Step 5: Sephadex G-200 chromatography

The enzyme solution was applied to a column (100 × 5 cm) of Sephadex G-200 equilibrated with 100 mM phosphate buffer pH 7.0 containing 2 mM F6P instead of ATP and eluted with the same buffer. PFK activity appeared in the first of three protein-containing peaks. The main fractions with PFK activity were pooled, and the enzyme precipitated by adding solid ammonium sulphate to a saturation of 0.45. After centrifugation at 26,000 *g*, the enzyme was redissolved in a small volume of phosphate buffer.

3.1.6. Step 6: Sepharose 4B chromatography

The enzyme solution was applied to a column of Sepharose 4B (70 × 2.5 cm) equilibrated with the same phosphate buffer as in step 5. During elution PFK activity appeared in several separate peaks [10]. The pooled main fractions were concentrated by precipitation of the enzyme with ammonium sulphate at 0.45 saturation. The precipitate was collected by centrifugation and redissolved in a minimum volume of 100 mM phosphate buffer pH 8.0.

A summary of a typical preparation of PFK from 2400 ml erythrocyte sediment is shown in table 1. The final specific activity of the purified enzyme was 136 units per mg protein. The purification factor was about 44,000 with 50% yield. The specific activities from other preparations ranged from 120–150 units per mg protein. As judged by SDS-electrophoresis, the final preparation appeared to be homogeneous. It showed only one band having a molecular weight of 104,000 under dissociating conditions [10].

3.2. *Some properties of the enzyme*

The purified enzyme (7–12 mg protein per ml) if stored in a medium containing 100 mM potassium phosphate (pH 8.0), 10 mM ammonium sulphate, 1 mM EDTA, 5 mM mercaptoethanol, 0.5 mM FDP, 4 mM 5′-AMP or 4 mM ATP is stable at 0° for at least 3 weeks.

Heat stability of the enzyme at step 4 was studied in a medium containing 100 mM potassium phosphate (pH 8.0), 1 mM EDTA, 5 mM mercaptoethanol, 0.5 mM FDP, 0.1 mM ATP and 0.25 saturation with ammonium sulphate. The results of these experiments are presented in fig. 1, A and B. In the purification procedure the results of these 2 experiments were combined leading to a high efficiency of the heat treatment.

Purified erythrocyte PFK has been found to be free of other glycolytic enzymes as well as of glucose-6-phosphate dehydrogenase, adenylate kinase, ATPase and NADH oxidase. At 1 mM F6P and 0.25 mM ATP the enzyme shows a broad pH-optimum between 7.6 and 8.4 (fig. 2). This is in agreement with the pH-activity curve of rat erythrocyte PFK [14].

4. Discussion

The procedure described allows the preparation of highly purified homogeneous PFK from human erythrocytes, devoid of other glycolytic enzyme activities and having a specific activity of 120–150. These values are comparable to those of homogeneous and crystallized preparations from other mammalian tissues. The crucial point of the present procedure seems to be the heat treatment of the enzyme (step 4). In the procedure of Layzer et al. [7] a considerable loss of enzymatic activity occurs upon heat treatment of the enzyme with a negligible increase in specific activity. In our experience, the results of heating the enzyme can be improved by addition of ammonium

sulphate and FDP. The optimal concentrations of these solutes have been tested and included in the purification scheme.

In Sepharose 4B chromatography the enzyme is eluted in several fractions. As has been shown, these differ in their molecular weights [10]. After cleavage of these forms with SDS, all of them can be traced back to a subunit having a molecular weight of 104,000 [10]. Until now, this SDS-cleavage product seems to be the common subunit of all oligomeric forms of erythrocyte PFK separable by Sepharose 4B chromatography.

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