

# THE ISOLATION OF ENZYME TRANSKETOLASE FROM HUMAN ERYTHROCYTES: THE CHAR- ACTERIZATION OF ITS QUARTERNARY STRUCTURE

ROSHANAK RAHIMIAN, Ph.D.,\* BIJAN FARZAMI, Ph.D., AND  
ABBAS SAMADI, Ph.D.

*From the Department of Biochemistry, School of Medicine, Tehran University of Medical Sciences, Tehran, and  
the Department of Pharmacology; School of Medicine, Gilan University of Medical Sciences, Gilan, Islamic  
Republic of Iran.*

## ABSTRACT

Human erythrocyte transketolase (sedoheptulose-7-phosphate: D-glyceraldehyde-3-phosphate, glycolaldehyde transferase, E.C. 2.2.1.1.) has been isolated from erythrocytes with a specific activity of 59.84 U/mg. SDS-PAGE and SE-HPLC were used both as a measure of purity and as a preparative mean to obtain a higher degree of purity. Four protein bands corresponding to molecular weights of 32,000, 39,000, 43,000 and 60,000 were obtained in electrophoresis and SE-HPLC preparations. Activity measurements on the two fractions obtained from SE-HPLC that contained a monomer with the molecular weight of 32,000 and a dimeric fraction with the molecular weight of 60,000 showed that the monomeric form of the enzyme displays activity in the presence and absence of the TPP and Mg(II). This activity was measured to be 14.76 U/mg in the absence of TPP and Mg(II), and 40.24 U/mg in the presence of the cofactors. The dimeric form showed an activity of 58.84 U/mg in the presence of the cofactors.

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## INTRODUCTION

Transketolase (E.C. 2.2.1.1.) is an enzyme of the pentose cycle which catalyzes ketal transfer to an aldehyde acceptor molecule and requires TPP and a divalent metal ion as cofactors.<sup>1</sup> Although Mg<sup>++</sup> is the physiological metal ion used in the enzymatic reactions, other divalent cations such as Ca<sup>++</sup> may also activate the enzyme.<sup>2,3</sup>

The enzyme has been obtained from several diffe-

rent sources. The baker's yeast transketolase is a dimer with a molecular weight of 159,000.<sup>4</sup> The enzyme obtained from pig liver is a tetrameric form with a molecular weight of 138,000.<sup>5</sup> The rat liver transketolase is active in its monomeric form and no apparent difference in activity is detected in the dimeric form in comparison with the monomeric form of the enzyme.

The dimer formation from its monomers could not be demonstrated in *in vitro* studies and it is suspected that the dimeric entity is formed at the site of the polypeptide biosynthesis in the presence of the cofactors.<sup>6</sup> Although the chemistry of thiamine has been the subject of intense studies,<sup>7</sup> the chemical mechanism of interaction of TPP with the enzyme is poorly understood. In the pig liver transketolase, two molecules of TPP are bound to the protein molecule and the metal ion has no effect on such interactions.<sup>5</sup>

Correspondence: B. Farzami, Ph.D., P.O.Box 14155- 5399  
Tehran, Iran. Tel: 6112773

Abbreviation: TPP, thiamine pyrophosphate; G-3-PDH, glyceraldehyde-3-phosphate; TPI, triose phosphate isomerase; DEAE-cellulose, diethylaminoethylcellulose; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; SE-HPLC, size exclusion high performance liquid chromatography.

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Baker's yeast transketolase contains two active sites with one site having lower affinity for TPP. In fact the binding of TPP with one site requires the presence of a divalent ion. Thesecond site also associates with TPP at high concentration of the metal ion; however, only one of the sites exhibits the catalytic activity.<sup>8</sup> Other evidences show that the other site contains a regulatory potential which can be activated by some molecules analogous to the substrates.<sup>9</sup> These evidences have been obtained for another thiamine-requiring enzyme i.e. pyruvate decarboxylase.<sup>10</sup>

The present study describes the isolation of transketolase from human erythrocytes and characterization of apoenzyme activity in its various forms.

### MATERIALS AND METHODS

**Materials:** Outdated blood was obtained from local blood banks. Transketolase, ribose-5-phosphate, TPP, L-cysteine hydrochloride, xylulose-5-phosphate, TPI,  $\beta$ -NADH, sedoheptulose-7-phosphate, G-3-PDH, DEAE-cellulose, tris-hydrochloride and TES were all purchased from Sigma Chemical Company, St. Louis, MO. Acrylamide, N,N-methylene bisacrylamide and glycine were obtained from Merck Chemical Company, Darmstadt, F.R.G. All other chemicals were reagent grade and purchased from a variety of sources. Water purified with a Millipore Mill-Q Water Purification System was used throughout the chromatographic separations.

**Preparation of the hemolysate:** A pack of one hundred milliliter of blood was centrifuged (10,000 Xg, 4°C) for 20 min. The supernatant was drawn off and the erythrocytes were washed three times with the physiological saline. After centrifugation, the packed cells were frozen at -20°C, and thawed. This procedure was repeated three times. Upon the last thaw, the hemolysate was diluted 1:1 with Tris buffer (50 mM, pH 8.0) containing 0.01% KCN and centrifuged at 11,000 Xg for 25 min. The transparent hemolysate was carefully collected and transferred to a constant volume dialysis chamber (PM 30 Ultrafiltration Membrane). The reservoir contained the same Tris buffer as above. The dialysis was continued until approximately 400 ml of the dialysate was collected. Concentration dialysis was carried out with 100 ml of the hemolysate in a dialysis bag (cut off point 12,000) under the vacuum pressure of 20 psi at 4°C. All of the preparative steps were carried out at 4°C.

**Chromatographic separation:** Approximately 250 g of DEAE-cellulose was suspended in one liter of Tris buffer (50 mM, pH 8.0) containing 0.01% KCN. The slurry was left at 4°C overnight. A chromatographic column (45X600 mm) is packed with 200 g of the resin and eluted with the same buffer several times until the

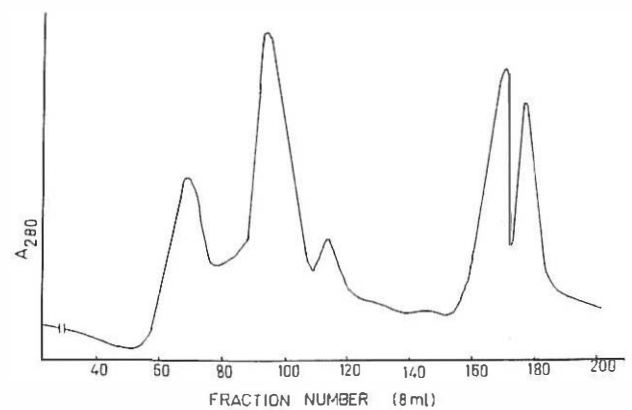


Fig.1. DEAE-cellulose ion exchange separation of human erythrocyte transketolase. DEAE-cellulose column (45x600mm) was equilibrated with Tris buffer (50 mM, pH 8.0, containing 0.01% KCN) overnight. The sample dialysate (50 ml) is packed with the resin on the top of the column and eluted with the same buffer devoid of 0.01% KCN. The flow rate was 1.5 ml/min without adjustment. Fractions (8.0 ml) were collected and analyzed for protein content (Lowry's method) and enzymatic activity.

eluent pH reached the pH of the buffer. The remaining resin was kept for use in the application of the sample over the column.

The sample (50 ml) of dialysate was added to the resin and the slurry was placed on the top of the DEAE-cellulose column, and eluted with Tris buffer (50 mM, pH 8.0) with the flow rate of 1.5 ml/min. The sample fractions 81-99 corresponding to the second peak, (Fig. 1) contained enzyme activity which were pooled and concentrated to about 15 ml using the concentration dialysis with a semipermeable membrane.

**Preparation of apoenzyme:** To the concentrated sample containing 60 mg of protein (3.1 mg/ml), 15 ml of saturated ammonium sulfate solution and 4.4 ml of 4 mM sodium acetate were added and the pH was adjusted to 3.5 with 1N sulfuric acid. The solution was centrifuged (35,000Xg 20 min), and the clear super-

Table 1. Purification of Human Erythrocyte Holoenzyme Transketolase

| Purification Steps                            | Protein Conc.(mg/ml) | Specific Activity (U/mg) |
|---|----------------------|--------------------------|
| 1. Concentration Dialysis                     | 137                  | 1.200                    |
| 2. DEAE-cellulose Ion Exchange (Two Fraction) | 1.7<br>1.9           | 1.32<br>1.48             |
| 3. Concentration                              | 3.1                  | 3.812                    |
| 4. Ammonium Sulfate precipitation             | 1.6                  | 6.3214                   |
| 5. SE-HPLC Purification                       | 0.02<br>0.04         | 59.84<br>40.24           |

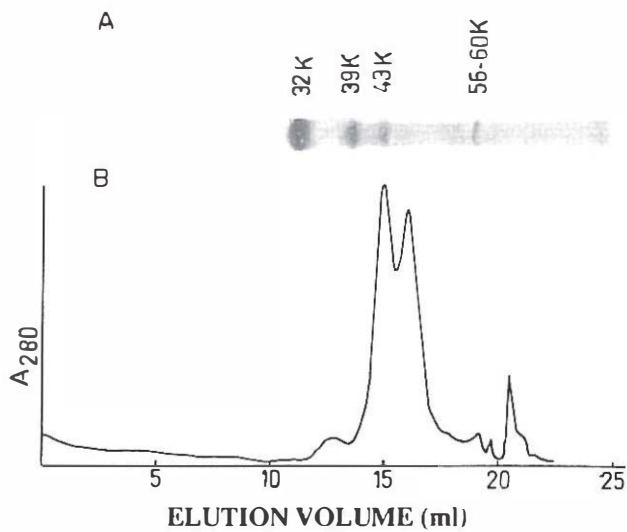


Fig.2. (A) SDS-PAGE, (B) SE-HPLC of apoenzyme transketolase.

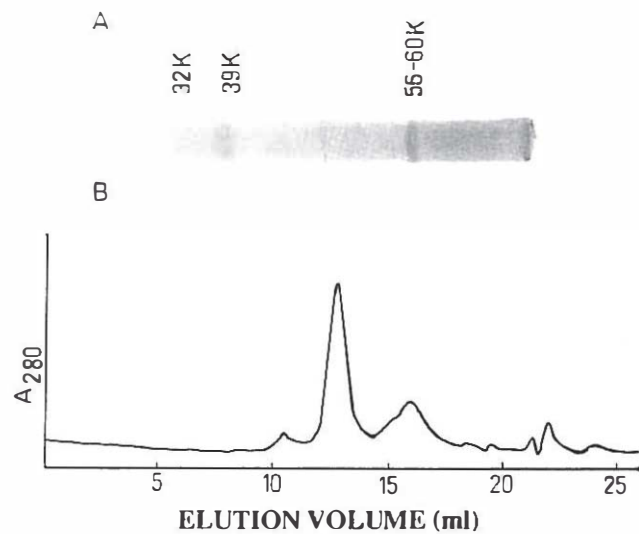


Fig.3. (A) SDS-PAGE, (B) SE-HPLC of holoenzyme transketolase.

nant was removed. The pellet containing the holoenzyme was kept at  $-20^{\circ}\text{C}$  in a desiccator. Half of the pellet was dissolved in 1.5 ml of TES buffer (0.5 M, pH 6.8). A homogeneous suspension was obtained which was centrifuged (35,000Xg, 10 min). The clear supernatant containing the apoenzyme was removed from the precipitate and stored at  $4^{\circ}\text{C}$  for further analysis.

**Activity measurement:** The enzymatic assay was carried out by Brin's method<sup>11</sup> with some modification, mixing 50-200  $\mu\text{l}$  of the specimen with 200  $\mu\text{l}$  of 50 mM TPP, 50 mM  $\text{MgCl}_2$  and 100  $\mu\text{l}$  of 0.1 M bicarbonate buffer, pH 7.5. After 15 min of incubation, 50  $\mu\text{l}$  of 220 mM ribose-5-phosphate was added to the mixture followed by one hour of incubation at room temperature. Twenty microliter of this mixture was added to an assay mixture containing 0.5 ml of 0.1 M bicarbonate buffer pH 7.5, 0.5 ml of 0.4 mM solution of  $\beta$ -NADH, 100  $\mu\text{l}$  of 20 mM xylulose-5-phosphate and 10  $\mu\text{l}$  of G-3-PDH/TPI (5 U/ml of each enzyme) and the rate of change in optical density at 340 nm was read against a blank containing all the reagents except the specimen. The enzyme assay was carried out at constant temperature of  $25^{\circ}\text{C}$  during the course of the reaction. The protein measurement was carried out using Lowry's method.<sup>12</sup>

**SDS-polyacrylamide gel electrophoresis:** Laemmli's method was used for the separation of proteins and the molecular weight determination of protein fractions.<sup>13</sup>

The apoenzyme protein fraction was separated (Laemmli's method) on 15% acrylamide, 0.8% bisacrylamid, 0.1% SDS gel. LMW protein standards (Pharmacia-LKB, Bromma, Sweden) were used as protein markers. The gels were stained with coomassie blue (0.2% coomassie blue, 20% acetic acid, 40% ethanol, 0.05% 2-mercaptoethanol) and destained

with 10% acetic acid, 40% ethanol.

**High performance liquid chromatography:** HPLC was carried out using size exclusion column TSK G2000SW (7.5 $\times$ 600 mm, LKB, Bromma, Sweden). The column was eluted isocratically with 0.1 M bicarbonate buffer (pH 6.8). For analytical studies, 20  $\mu\text{l}$  of the protein mixture was injected to the TSK G2000SW column. The preparative separation of the protein fractions were carried out using 200  $\mu\text{l}$  sample loop. The absorbance was monitored at 280 nm with a full-scale deflection of 0.065 absorbance unit.<sup>18-20</sup> The corresponding fractions were collected, dialysed against distilled water and lyophilized and stored at  $-20^{\circ}\text{C}$ .

## RESULTS AND DISCUSSION

This report presents a highly purified enzyme with fixed physical properties as indicated by SDS-PAGE and HPLC methods. The purification fold as indicated in Table I was 60 at the final stage with the specific activity of 64 U/mg. But previous methods of isolation of transketolase from human erythrocytes contained either impurities of glyceraldehyde-3-phosphate<sup>14</sup> or were partially purified.<sup>15</sup> The SDS-PAGE showed four bands with the molecular weights of 32,000; 56-60,000; 39,000 and 43,000 in apoenzyme and three bands with the molecular weights of 32,000; 58-60,000 and 39,000 in holoenzyme. The gel contained few faint bands indicating small impurities that were mainly eliminated in the later stages of purification using HPLC. The fractions obtained from HPLC contained three bands both in holoenzyme and in apoenzyme (Fig. 2,3). In apoenzyme two new bands were with different reten-

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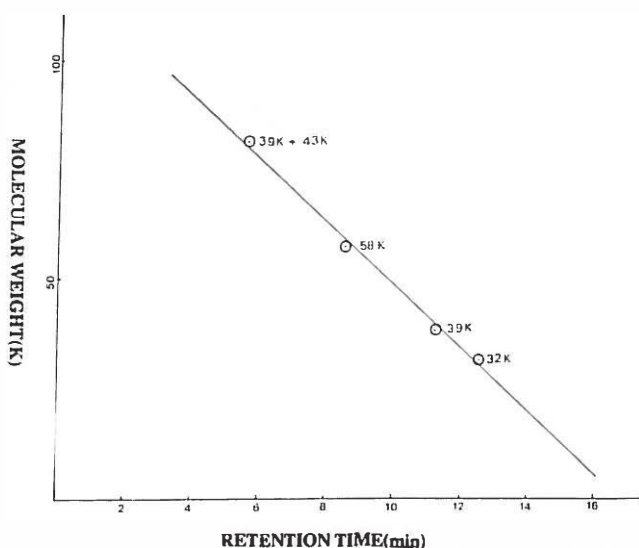


Fig. 4. Relationship between the molecular weight determination by SDS-PAGE and information obtained from SE-HPLC. See figures 2 and 3.

tion times and intensities (Fig. 2). The fractions obtained by these methods were compared with SDS-PAGE results and strict linear correspondence was observed when the molecular weights obtained from SDS-PAGE were plotted against the retention times observed by SE-HPLC (Fig. 4). Therefore, a peak corresponding to the molecular weight of 82,000 was detected in holoenzyme fraction which was not present in the apoenzyme. This band was apparently due to a dimeric form of the enzyme formed from the monomeric forms of molecular weights 39,000 and 43,000 which was observed both in SDS-PAGE and SE-HPLC. In SDS-PAGE, the band corresponding to 43,000 was not present in holoenzyme indicating that it participates in the structure of holoenzyme. In SE-HPLC method, the two bands corresponding to molecular weights of 32,000 and 39,000 were present from which the peak with the molecular weight of 39,000 was adjoined to the structure of holoenzyme.

From the peaks obtained from SE-HPLC, it is apparent that a dimer with a molecular weight of 58-60,000 has a lower stability than the dimer formed from the monomers of molecular weights of 39,000 and 43,000 since the separation of the former took place more distinctly in the apoenzyme form. This observation is also substantiated in SDS-PAGE since the peak corresponding to 43,000 disappeared more readily with the addition of equimolar amounts of TPP and  $Mg^{++}$  to the apoenzyme solution.

The difference in the specific activity of the apoenzyme from the holoenzyme is indicative of the role of the cofactors in the enzyme activation. Previous reports emphasize the role of cofactors in catalytic functions of the enzyme.<sup>6,8,16</sup> The results obtained using

SE-HPLC method indicates that the 60,000 fraction is more active than the monomeric forms in the presence of cofactors. The existence of tetrameric form of the enzyme was not detected in these experiments. The intensities of each of the dimeric forms as fractions obtained in SE-HPLC and SDS-PAGE indicate that the dimeric form with the molecular weight of 82,000 constitutes only 7-10% of the fraction with the molecular weight of 58-60,000 and therefore can be accounted mainly as a separate form of the enzyme. It is noteworthy that total molecular weight of the dimeric forms is 138-140,000 corresponding to the molecular weight of the tetrameric form of the enzyme isolated from pig liver.<sup>17</sup> Although a close correspondance exists between the molecular weight of the tetrameric forms of the enzyme obtained from the pig liver and the total weights of the dimers obtained in our study no indication of the existence of this tetrameric form is observed in our experiments.<sup>17</sup> Furthermore, the difference in the proportions of these two forms in our isolated enzyme is a supporting evidence that the enzyme exist, in our preparations, of two dimeric forms with the molecular weights of 58-60,000 and 82,000 belonging to two isomeric forms of the enzyme.

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