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Note

Separation of deamidated forms of triosephosphate isomerase by chromatofocusing

A comparison of chromatofocusing with column isoelectric focusing

BEDII ORAY, K. ÜMIT YÜKSEL and ROBERT W. GRACY*

Department of Biochemistry, North Texas State University, Denton, TX 76203 (U.S.A.) and The Texas College of Osteopathic Medicine, Fort Worth, TX 76107 (U.S.A.)

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Triosephosphate isomerase (TPI, E.C. 5.3.1.1) catalyzes the interconversion of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. The enzyme is a dimer (molecular weight, M_r 53,000) of identical subunits[1]. TPI is found in virtually all tissues of most species[2,3] and has been purified and characterized from several sources[4–7]. The electrophoretic multiplicity reported by several laboratories[5,8,9] has been explained by the formation of anodal subforms of TPI both *in vitro*[10] and *in vivo*[11] which are thought to be the result of spontaneous deamidation of two asparagines (Asn-15 and Asn-71)[12]. However, more direct evidence for the deamidation process is needed, and preparative methods for the isolation of the multiple forms are essential for the further study of the deamidation process.

Sluyterman and co-workers[17,18] described both theoretically and experimentally the possibility of producing focusing effects in ion-exchange columns in a pH gradient similar to those in isoelectric focusing. They also reported that, in the comparison of chromatofocusing with column isoelectric focusing, the resolution power is somewhat greater in the chromatofocusing procedure.

We report herein the application of chromatofocusing in the separation of deamidated forms of TPI and a comparison of chromatofocusing with preparative (column) isoelectric focusing. Advantages and disadvantages of both procedures are discussed.

MATERIALS AND METHODS

Materials

Polybuffer 74 and PBE 94 for chromatofocusing were purchased from Pharmacia. All reagents for isoelectric focusing were obtained from LKB, while polyacrylamide gel electrophoresis reagents were from Bio-Rad Labs. Enzymes, substrates and coenzymes were purchased from Sigma. All other chemicals and solvents were at least analytical-reagent grade and were further purified by recrystallization or redistillation.

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Enzyme isolation

Rabbit muscle TPI was purified according to the methods described previously[13,14]. The enzyme was judged to be homogeneous by sodium dodecyl sulfate (SDS) gel electrophoresis. Enzyme assays were performed at 30°C using glyceraldehyde 3-phosphate as substrate[14]. The enzyme preparations used in this study were stored at 4°C for up to 1 year.

Chromatofocusing

Chromatofocusing experiments were conducted on a 30 × 0.9 cm (Type K9/30, Pharmacia) column packed with PBE 94 and equilibrated with 25 m*M* imidazole hydrochloride (pH 7.0). After the addition of *ca.* 13 mg of TPI (which was first dialyzed against the initial imidazole buffer), the column was washed with 5 ml of the same buffer. After this step, the column was washed with Polybuffer 74 (1:10 diluted, pH 6.0) to form the pH gradient. Flow-rates were adjusted with a LKB Model 2120 Varioperpex II pump. The UV absorption at 280 nm was monitored with a CE 212 variable-wavelength UV monitor (Cecil Instruments). Fractions (1.25 ml) were collected and analyzed.

Isoelectric focusing

Preparative isoelectric focusing experiments were carried out using 2% ampholines (pH 5–8) and a sucrose density gradient in a LKB 8100 column at 4°C at 450 V for 72 h. At the end of the experiment, fractions were collected and analyzed.

Polyacrylamide gel electrophoresis

Alkaline disc slab gels (7.5% resolving gel/3% stacking gel) were prepared in a vertical slab gel cell (Bio-Rad Model 220) according to the method of Maizel[15]. Electrophoresis was carried out at 30 mA constant current until the tracking dye reached the bottom of the gel. TPI activity was located with the coupled formazan precipitation stain originally described by Scopes[16] and later modified by Snapka *et al.*[3]. Protein staining was with Coomassie Brilliant Blue R stain. Polyacrylamide gel electrophoresis in SDS was conducted in a phosphate buffer system[15] with tube format (10 × 0.5 cm). Electrophoresis was carried out for *ca.* 6 h at room temperature at 5 mA per gel until the tracking dye migrated to the bottom of the tube. Staining and destaining procedures were the same as for the alkaline disc slab gel system described above.

RESULTS AND DISCUSSION

In the study of spontaneous deamidation of enzymes such as TPI, it is very important to utilize a simple and rapid method to separate the individual deamidated forms from the native enzyme. These multiple forms represent proteins with single charge changes and the method to be used must be powerful enough to resolve these multiple forms yet gentle enough to preserve catalytic activity.

A series of studies utilizing chromatofocusing and electrofocusing was conducted and representative results are presented below. As shown in Fig. 1, chromatofocusing can be utilized to separate the subforms of the enzyme. Fractions which contain the different forms of the enzymes, were then subjected to alkaline disc slab

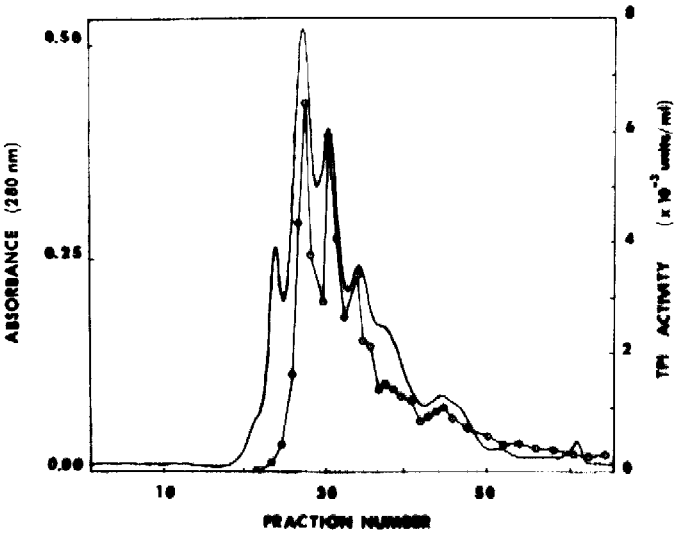

Fig. 1. Chromatofocusing of a 1-year-old, pure (homogenous) TPI preparation. The solid line is the absorbance at 280 nm; ○, TPI activity (units/ml). The TPI (13 mg) was loaded on a 30 × 0.9 cm column packed with PBE 94. The pH gradient formed was 7.0 6.0. Experimental details are described in the Materials and methods section.

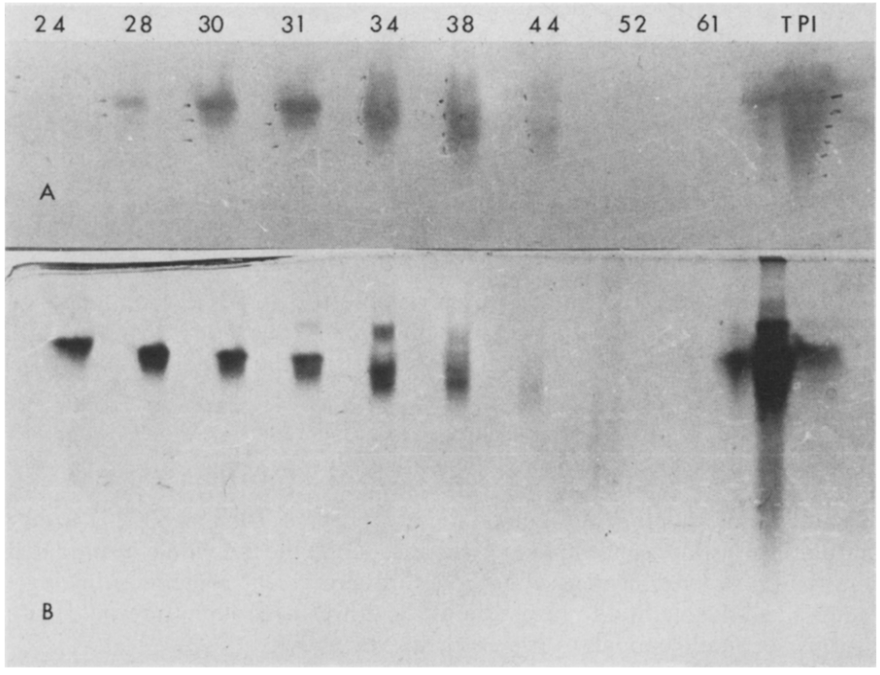

Fig. 2. Alkaline disc slab gel electrophoresis of a 1-year-old, pure (homogenous) TPI. A, TPI activity stain; B, protein staining with Coomassie Brilliant Blue. The lane numbers refer to fraction numbers in Fig. 1. The lane marked TPI is an unfocused sample. Specifics of electrophoresis and staining are given in the Materials and methods section.

gel electrophoresis. The results of the electrophoresis experiments are shown in Fig. 2. Fig. 2B shows the results of protein stain, and Fig. 2A the TPI catalytic activity stain. Since each fraction from the chromatofocusing column exhibited unique electrophoretic mobility on alkaline disc gel electrophoresis, it was concluded that the chromatofocusing does indeed separate the different forms of the enzyme, and that most of the resolved forms still possess catalytic activity.

When identical enzyme preparations were subjected to preparative (column) isoelectric focusing, as shown in Fig. 3, the deamidated forms of TPI were also resolved. In fact, in this case the resolution of the subforms was somewhat superior to that obtained by chromatofocusing.

In the case of resolving the isoenzymes of TPI which differ by only one to four charge changes, both of these procedures seem to be satisfactory. Column isoelectric focusing requires much longer time (up to 72 h), but the separation of the subforms is somewhat superior. On the other hand, column isoelectric focusing requires more sophisticated, expensive instrumentation. If the initial capital cost of instrumentation is excluded, both procedures cost about the same per experiment. The yield of catalytically active enzyme from both procedures was essentially the same and varied between 50 and 65%.

In conclusion, when separation of proteins on a preparative scale is needed (even the subforms of the same protein), both chromatofocusing and isoelectric focusing can be utilized. Isoelectric focusing still seems to exhibit better resolving power, but requires more sophisticated instrumentation and the resolution time is much longer.

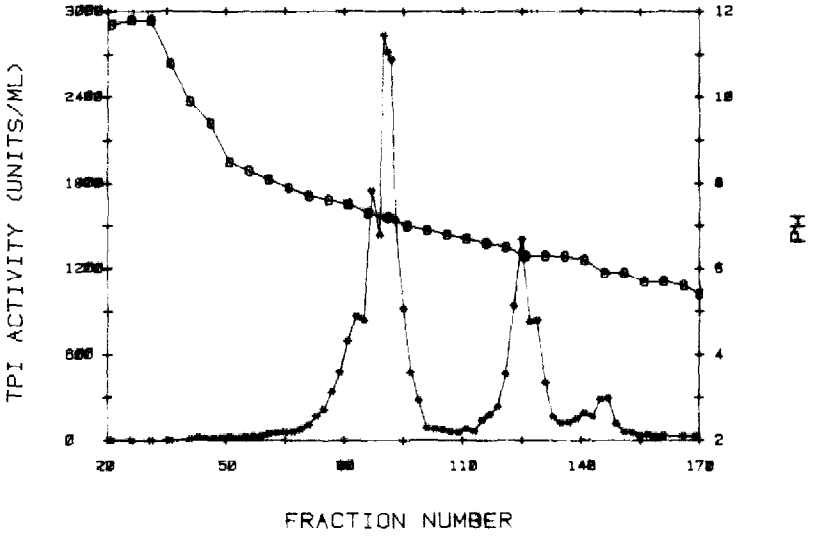

Fig. 3. Preparative isoelectric focusing of a 1-year-old, pure (homogenous) TPI preparation. Focusing was carried out in a LKB 8100 column using 2% ampholines (pH 5–8) and a sucrose gradient at 4°C at 450 V for 72 h. *, TPI activity (units/ml); ○, pH.

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