Journal of Chromatography, 490 (1989) 424–431
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4692

Note

Purification of human muscle phosphoglycerate mutase by fast protein liquid chromatography based on hydrophobic interactions

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(First received October 17th, 1988; revised manuscript received January 18th, 1989)

In the serum of patients suffering from Duchenne muscular dystrophy (DMD) a number of muscle-specific isoenzymes and other proteins have been shown to be greatly increased above normal levels. These include muscle creatine kinase [1], muscle pyruvate kinase [2], carbonic anhydrase III [3] and myoglobin [4]. Phosphoglycerate mutase (PGAM) is a dimeric enzyme; the subunits may be either the muscle (M) or brain (B) type, so that three possible isoenzymes may be formed, i.e. MM, BB, or MB. The predominant PGAM isoenzyme in skeletal muscle is the MM form [5]. Isoelectric focusing of serum samples from normal subjects and from patients with DMD showed that only the serum samples from dystrophic patients contained the MM isoenzyme whilst all serum samples contained BB-PGAM [6]. This explains why a measurement of total serum PGAM activity from dystrophic patients showed only a relatively small elevation above the normal activity [7]. However, an assay for MM- or M-chain PGAM is likely to reflect, more accurately, the degree of elevation of the muscle PGAM, as interference from the non-muscle-derived PGAM would be eliminated. Such an assay would be useful in adding to the information of the extent and nature of the leakage of muscle proteins into the

0378-4347/89/$03.50

serum of patients with various forms of muscular dystrophy, polymyositic and other degenerative diseases of the muscle. To this end, a specific and sensitive immunoassay is being developed. However, it is first necessary to purify human MM-PGAM so that antibodies can be raised against it. The successful purification of this isoenzyme using a combination of ion-exchange and hydrophobic interaction chromatography is described. The purification of the human enzyme had not previously been reported.

EXPERIMENTAL

Materials

Fast protein liquid chromatography (FPLC) apparatus, the pre-packed chromatographic columns, MonoQ, MonoS, Superose 12 and Phenyl-Superose, and Phenyl-Sepharose Fast Flow were obtained from Pharmacia (Milton Keynes, U.K.). Hydroxyapatite was purchased from Bio-Rad (Watford, U.K.). Diethylaminoethylcellulose (DEAE), DE52, was supplied by Whatman (Maidstone, U.K.). All chromatography columns were from Amicon (Stonehouse, U.K.). The electrophoresis reagents were obtained from National Diagnostics (Aylesbury, U.K.). All chemicals were purchased from Sigma (Poole, U.K.) or BDH (Poole, U.K.) and were of AnalaR grade or the best equivalent.

Extraction of muscle proteins and initial ion-exchange chromatography

Human muscle was obtained post-mortem within 48 h after death, stripped of connective tissue, minced and homogenised with 2 volumes of ice cold distilled water containing 2 m*M* EDTA, 1 m*M* 2-mercaptoethanol and 2 m*M* phenylmethylsulphonyl fluoride. The homogenate was stirred on ice for 10 min before centrifugation at 10 000 *g* for 20 min at 4 °C. The supernatant was exhaustively dialysed against 10 m*M* Tris–HCl, 1 m*M* 2-mercaptoethanol, pH 8.5 and then applied, at a flow-rate of 3.0 ml/min, to a column of DE52 (45 cm × 3.0 cm), previously equilibrated in the same buffer. PGAM did not bind to the column. The fractions containing PGAM activity were pooled and solid ammonium sulphate was added to 80% saturation to stabilise enzyme activity. Before further purifications using ion-exchange or hydroxyapatite chromatography were attempted, the preparation was dissolved in the appropriate buffer, as stated below, and dialysed three times against 100 volumes of the same buffer at 4 °C. Before chromatography by hydrophobic interaction chromatography the preparation was diluted with distilled water to bring the concentration of ammonium sulphate to approximately 2 *M*.

Other chromatographic procedures

The FPLC apparatus consisted of an LCC-500 chromatography controller, two P-500 high-precision pumps, an MV-7 motor-driven injection valve, a 10-ml Superloop, a UV-1 (280 nm) monitor, a REC-482 two-pen recorder and a

FRAC-100 fraction collector (all apparatus was from Pharmacia). The chromatographic columns used were MonoQ HR5/5, MonoS HR5/5, Superose 12 HR10/30 and Phenyl-Superose HR5/5. Anion-exchange chromatography on MonoQ was performed by a 20-min linear gradient from 0 to 0.35 *M* NaCl, and buffered with 20 m*M* ethanolamine (pH 9.0 or 9.5) or 20 m*M* diethanolamine (pH 10.0). Cation-exchange chromatography on MonoS was performed by a 20-min linear gradient from 0 to 0.50 *M* NaCl, and buffered with 50 m*M* 2-(N-morpholino)ethanesulphonic acid (pH 6.0) or 50 m*M* sodium phosphate (pH 7.0). In all cases the flow-rate was 1.0 ml/min. Gel-permeation using Superose 12 was performed in 50 m*M* sodium phosphate, 0.15 *M* NaCl, pH 7.5, at a flow-rate of 0.05 ml/min. For hydrophobic interaction chromatography using Phenyl-Superose two solutions were made: buffer A (50 m*M* potassium phosphate, pH 7.0) and buffer B (50 m*M* potassium phosphate, 1.7 *M* ammonium sulphate, pH 7.0). After equilibration of the column in buffer B, the sample was loaded and then eluted using a sequential gradient of 100% B for 5 min, 100–60% B over 5 min, followed by 60–40% B over 30 min and finally 40–0% B over 5 min. The flow-rate was 0.5 ml/min throughout.

Hydroxyapatite chromatography was performed in a conventional low-pressure glass chromatography column, packed to 6.5 cm × 1.0 cm, and the FPLC instrument was used to generate a linear gradient of 50 to 250 m*M* sodium phosphate over 25 ml at 1.0 ml/min. Phenyl-Sepharose Fast Flow was packed to 5.0 cm × 1.0 cm and the elution gradient applied and buffers used were the same as those used with Phenyl-Superose.

Analysis of PGAM preparations

Protein determinations on crude samples were performed following the method of Bradford [8]. Protein levels in pure PGAM samples were determined using an absorption coefficient of 1.4 at 280 nm for a solution of 1 mg/ml. PGAM activity was determined using the enolase coupled assay of Grisolia and Carreras [9]. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 10% resolving gels with 2% stacking gels, as described by Laemmli [10].

RESULTS

The first chromatography step used anion-exchange on DE52. The PGAM preparations produced contained two major contaminants (Fig. 1) with M_r 60 000 (contaminant A) and M_r 45 000 (contaminant B). All of the other contaminants were easily removed by further ion-exchange chromatography, but neither PGAM nor the two contaminants would bind to either MonoQ or MonoS columns at pH 9.5 or 7.0, respectively, and further extremes of pH caused denaturation of PGAM.

Chromatography using hydroxyapatite resulted in removal of contaminant

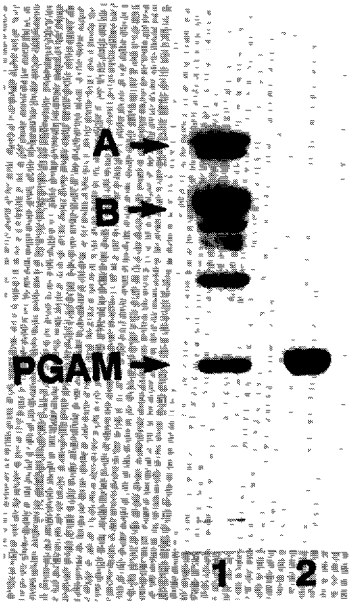

Fig. 1. SDS-PAGE of a human muscle extract following DEAE-cellulose chromatography (lane 1); 30 μg of protein were applied to the gel. The bands corresponding to PGAM, contaminant A and contaminant B are indicated; M_r values were 29 000, 60 000 and 45 000, respectively. In lane 2 the same human muscle extract is shown after further purification by Phenyl-Superose (see Experimental for details); 10 μg of protein were applied to the gel. This preparation was completely separated from all minor contaminants and the two major contaminants that were present to a varying degree in all human muscle samples used for the purification of PGAM.

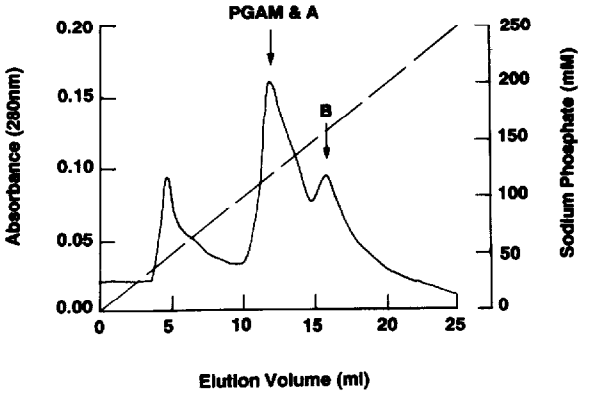

Fig. 2. Hydroxyapatite chromatography of a human muscle extract. The preparation applied to the column (approximately 1 mg) was obtained from DEAE-cellulose chromatography as described in the Experimental section. The broken line shows the gradient of increasing sodium phosphate concentration applied to the chromatographic column. The elution positions of PGAM and contaminants A and B are shown.

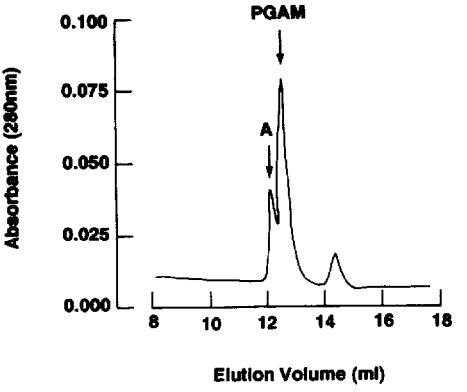

Fig. 3. Gel-permeation chromatography of a human muscle extract using Superose 12. The preparation applied to the column (approximately 0.1 mg) was obtained from DEAE-cellulose and hydroxyapatite chromatography (see Fig. 2). The elution positions of PGAM and contaminant A are shown.

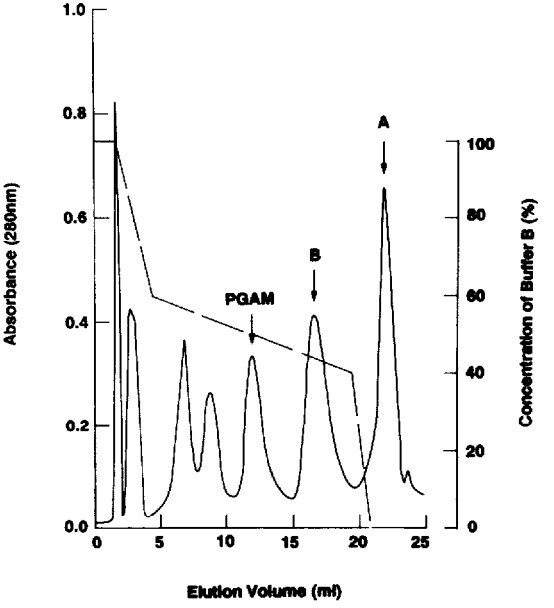

Fig. 4. Hydrophobic interaction chromatography of a human muscle extract using Phenyl-Superose. The preparation applied to the column (approximately 4 mg) was obtained from DEAE-cellulose chromatography as described in the Experimental section. The broken line shows the gradient of decreasing concentration of buffer B (50 m*M* potassium phosphate, 1.7 *M* ammonium sulphate) applied to the chromatographic column. The elution positions of PGAM and contaminants A and B are shown.

TABLE I

TYPICAL PURIFICATION OF HUMAN MUSCLE PHOSPHOGLYCERATE MUTASE EXTRACTED FROM HUMAN MUSCLE, USING ION-EXCHANGE CHROMATOGRAPHY AND HYDROPHOBIC INTERACTION CHROMATOGRAPHY

Fraction	Protein (mg)	Total activity (U)	Specific activity (U/mg)	Fold purification	Yield (%)
Muscle homogenate	219.0	3942	18	1.0	100
After DEAE-cellulose	24.0	3288	137	7.6	83
After Phenyl-Superose	8.8	1973	225	12.5	50

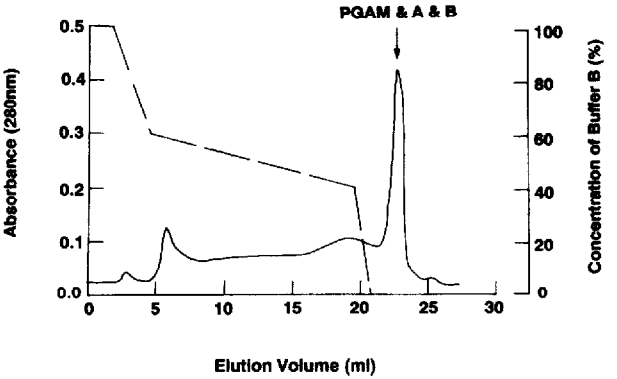

Fig. 5. Hydrophobic interaction chromatography of a human muscle extract using Phenyl-Sepharose Fast Flow. The preparation applied to the column (approximately 1 mg) was obtained from DEAE-cellulose chromatography as described in the Experimental section. The broken line shows the gradient of decreasing concentration of buffer B (50 m*M* potassium phosphate, 1.7 *M* ammonium sulphate) applied to the chromatographic column. The elution positions of PGAM and contaminants A and B are shown.

B from PGAM (Fig. 2). PGAM was then separated from contaminant A by gel permeation on Superose 12 (Fig. 3). However, only analytical quantities could be purified in this way as loading volumes larger than 0.2 ml resulted in a loss of resolution.

Separation by Phenyl-Superose had none of these problems. Electrophoretically pure PGAM (Fig. 1) with a specific activity of about 225 U/mg of protein was produced in a single step with a clear separation from both of the major and all the minor contaminants (Fig. 4). Volumes of up to 10 ml could be applied using the Superloop, provided that the amount of protein loaded was less than 5 mg. A typical purification is shown in Table I. Starting specific activities of the muscle homogenates varied between 6 and 30 U/mg of protein, being generally higher in the samples obtained with the least delay after death. The purification shown in Fig. 4 was for one of the poorest muscle samples

obtained: the muscle homogenate had a specific activity of only 6 U/mg of protein; nevertheless, a successful purification was still achieved (Fig. 1).

An attempt to scale up the process using Phenyl-Sepharose Fast Flow under the same elution conditions was attempted (Fig. 5). The result was a completely different profile compared with Phenyl-Superose (Fig. 4). PGAM and the two major protein contaminants were not resolved as they bound more avidly to Phenyl-Sepharose Fast Flow compared with binding to Phenyl-Superose under the same conditions.

DISCUSSION

Hydrophobic interaction chromatography provides an alternative means of purification of proteins and has been especially useful here for the purification of PGAM. The method, using a Phenyl-Superose column, proved to be fast, reproducible and thus superior to the more commonly used methods of hydroxyapatite and gel-permeation chromatography for the purification of this enzyme [11]. FPLC was able to purify only about 1–2 mg in a single chromatographic procedure, but the fast chromatography time (45 min) allowed repeated injections to be made onto the column and 10 mg could easily be purified in a single day. Theoretically, the quantity purified each time could be increased eight-fold by use of a larger, Phenyl-Superose HR10/10 column. An attempt to increase the quantity of PGAM purified using a Phenyl-Sepharose Fast Flow column under the same elution conditions was not successful as PGAM was not separated from the major contaminants. It is possible that a separation might be achieved under different elution conditions. However, the difference in the properties of Phenyl-Superose and Phenyl-Sepharose Fast Flow indicates that the hydrophobic interaction of the proteins with these chromatography materials is not limited to the phenyl groups.

This is the first report of a successful purification of human PGAM. The method was rapid, using only two purification procedures, and had a relatively high yield (50%) compared with the purification of muscle PGAM from the muscle of pig and other species, where typical yields were 28% after four separation procedures [11]. The improved yield was obtained even where the quantity available was small and the quality was poor. Sufficient quantities of PGAM for antibody production were obtained. The method of hydrophobic interaction chromatography thus provides a suitable means to purify this basic protein and retain enzyme activity.

ACKNOWLEDGEMENTS

We thank Mr. Peter Simpson (Pharmacia, Milton Keynes, U.K.) for helpful advice and the use of the Phenyl-Superose column. Financial support from St.

George's Hospital Special Trustees and the Muscular Dystrophy Group of Great Britain is gratefully acknowledged.

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