Journal of Chromatography, 491 (1989) 61–68
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4705

HIGH-PERFORMANCE ION-EXCHANGE CHROMATOGRAPHY OF MYOSIN USING A DEAE-5PW COLUMN

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(First received May 19th, 1987; revised manuscript received January 27th, 1989)

SUMMARY

High-performance ion-exchange chromatography of myosin using a DEAE-5PW packing was used to purify myosin from skeletal, cardiac and smooth muscle. This method produces high-speed resolution (30-min analysis) of myosin from contaminating myofibrillar proteins. The column has a high capacity for binding myosin (up to 1 g) and can be used for small-scale preparation of highly purified myosin. Gel analysis in the presence of sodium dodecyl sulfate showed recovery of myosin with very little contamination of other myofibrillar proteins. Myosin was also recovered from small biopsy samples (0.1 g) by a direct extraction technique with recovery of biological ATPase activity.

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INTRODUCTION

The isolation of protein from muscle is an important part of many physiological and biochemical studies. Myosin isolation from muscle is based on the solubility of the protein at high ionic strength and its insolubility at low ionic strength [1,2]. Chromatographic techniques are usually applied as the last step in the preparation of myosin to remove actin and regulatory proteins [3], or C-protein [4] if the myosin is to be used to raise antibodies. DEAE-Sephadex A-50 chromatography with a gradient of potassium chloride has been used to remove contaminating proteins in the 90 000 to 150 000 relative molecular mass range. However, column chromatography has several disadvantages: (a) eluted myosin has to be concentrated, thus prolonging the preparation of myosin; (b) chromatography is performed using an alkaline pH (8.5–9.0), which can destabilize some myosin isoform conditions; (c) column chromatography is not applicable to very small sample sizes (0.1 g).

This paper describes an application using ion-exchange high-performance liquid chromatography (HPLC) with a DEAE-5PW packing for the myosin preparation.

EXPERIMENTAL

Materials

The materials were obtained from the following suppliers: DEAE-Sephadex A-50 from Pharmacia Fine Chemicals (Piscataway, NJ, U.S.A.); DEAE-5PW ion-exchange HPLC column from Waters Chromatography Division (Milford, MA, U.S.A.); acrylamide, bisacrylamide sodium dodecyl sulfate (SDS), Tris base and glycine from Bio-Rad (Richmond, CA, U.S.A.); leupeptin, pepstatin and phenyl methane sulfonyl fluoride (PMSF) from Sigma (St. Louis, MO, U.S.A.). All other reagents were Analar grade obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.).

Preparation of myosin

Rabbit back, soleus, semitendinous, left ventricle and uterus muscles, rat psoas and uterus muscles and biopsied human ventricular and uterus samples were homogenized and the myosin was extracted as previously described [5,6]. After initial homogenization of the muscle in the presence of 0.2 μg/ml pepstatin, 0.2 μg/ml leupeptin, 1 m*M* EDTA and 0.1 mg/ml PMSF to inhibit protease activity, myosin extract was then subjected to ammonium sulfate precipitation between 32 and 38% (w/v) saturation. The resulting precipitate was collected by centrifugation at 10 000 *g* and then dialyzed overnight in 50 m*M* sodium pyrophosphate–1 m*M* EDTA, pH 7.5 at 4 °C, to remove the ammonium sulfate. Myosin was further fractionated to remove contaminating actin and C-protein either by passage through a DEAE-Sephadex A-50 column described

by Offer et al. [3] or by high-performance ion-exchange chromatography using a DEAE-5PW column. For conventional DEAE-Sephadex A-50 chromatography the column (30 cm×2.5 cm) was equilibrated in 50 m*M* sodium pyrophosphate, 1 m*M* EDTA, pH 7.5 at 4°C, with 10 column volumes. The sample was loaded onto the column at a flow-rate of 1.0 ml/min. Unretained material was washed from the column by elution with 5–10 column volumes of the above buffer. Myosin was then eluted from the column with a 500-ml linear gradient of 0–0.6 *M* potassium chloride in the above buffer, with the protein eluting at about 0.25 *M*.

Ion-exchange column chromatography

A Protein Pak DEAE-5PW column (7.5 cm×0.75 cm) was used with a Waters HPLC system consisting of a U6K injector, two Model M6000A pumps and a Model 440 multi-wavelength detector set at 280 nm. The inlet side of the column was fitted with additional tubing to act as a pre-column cooling coil. Then the column and cooling coil were immersed in an ice bath at 0±2°C. The column was equilibrated at 1.0 ml/min with a solution containing 50 m*M* sodium pyrophosphate, 1 m*M* EDTA, pH 8.8, which had been degassed and filtered through a 0.45-μm membrane filter (type HA, Millipore). The sample, which had been dialyzed against the above buffer in volumes up to 2 ml, was then loaded through the injection valve into a sample loop and introduced onto the column. Unretained material was eluted with the above buffer for 10 min. The protein adsorbed on the column was eluted with a 20-min linear gradient of 0–0.6 *M* potassium chloride in the above buffer. At the end of the gradient the column was flushed with 1.0 *M* potassium chloride in the above buffer for 10 min prior to returning to initial conditions for the next sample.

An alternative method was employed using less equipment and the results were compared with the aforementioned two-pump method. A Protein-Pak DEAE-5PW column (7.5 cm×0.75 cm) was used with a Waters HPLC system consisting of a U6K injector, one M45 pump with a manifold adapter, one Model 441 detector with a 280-nm aperture and one Omniscribe chart recorder (Houston Instruments). The column and coils were immersed in an ice bath at 0±2°C. The column was then equilibrated with 20 m*M* Tris–HCl, pH 7.6 at 4°C for 30 min at a flow-rate of 1.0 ml/min. Myosin extracts (0.5–2.0 ml) were injected into the sample loop and retained material was eluted in the above Tris buffer for 10 min at a flow-rate of 1.0 ml/min. The protein was eluted from the column by changing to a 20 m*M* Tris–HCl solution containing 0.6 *M* potassium chloride, pH 7.6 at 4°C, and producing a step gradient with a flow-rate of 0.8 ml/min for approximately 35 min.

Fractions from both methods were collected in chilled tubes and immediately placed in ice. All samples were stored at −20°C until electrophoretic and enzymatic analysis was conducted.

Extraction of myosin from biopsy samples

Human ventricular muscle biopsies (20–30 mg) were cut into small pieces and the myosin was extracted in 30 volumes of 20 m*M* sodium pyrophosphate in 10% glycerol (w/v), pH 8.8, overnight a 4 °C. The myosin extract was clarified by centrifugation at 100 000 *g* for 5 min. The protein content of the supernatant was estimated by the method of Lowry et al. [7]. A small volume of the myosin extract was then loaded onto the DEAE-5PW column and equilibrated as described above. The column was washed with 5 volumes of buffer before changing mobile phases to a solution containing 20 m*M* Tris–HCl, pH 7.6, in order to remove phosphate from the system. Myosin was eluted from the column with a linear gradient up to 0.6 *M* potassium chloride in the above Tris buffer. An ATPase assay was performed on the myosin eluted from the column using a method described by Sreter et al. [8].

One-dimensional SDS gel electrophoresis

Fractions from the above chromatographic separations were analysed by SDS gel electrophoresis using a high-resolution gel system [9] which produces sharper resolution of myofibrillar protein bands when compared to conventional procedures [10]. Samples (10–20 μg protein) were dissolved in a small volume of a solution containing 1.6% lithium dodecyl sulfate, 80 m*M* Tris–HCl, 6.0% glycerol (w/v), 80 m*M* mercaptoacetic acid and 0.001% bromophenol blue, pH 6.8. After heating to 100 °C for 2–3 min, the samples were then applied to a 0.75 mm thick 10% stacking gel (acrylamide–bisacrylamide, 37:1) in 125 m*M* Tris–HCl, pH 6.8, 0.1% SDS. The separating gel contained 16% acrylamide (150:1) in 750 m*M* Tris–HCl, 0.1% SDS, pH 8.8. The upper gel buffer (25 m*M* Tris base, 190 m*M* glycine and 0.1% SDS) contained 3 m*M* mercaptoacetic acid and 3 m*M* Tris base. The lower gel buffer had the same composition as the upper buffer except that SDS and mercaptoacetic acid were omitted. Electrophoresis was carried out at 30–40 mA per gel at constant current for 2 h. Staining and destaining were carried out as described earlier [9].

Protein assay on column fractions

Protein concentration in the column chromatographic fractions was measured using the protein procedures of Lowry et al. [7].

RESULTS

Myosin extract after ammonium sulfate precipitation still contains significant amounts of actin and C-protein. The established procedure to remove these contaminants involves passing the sample through a DEAE ion-exchange column as reported by Offer et al. [3].

Rabbit skeletal white (fast) muscle myosin was eluted using both conventional DEAE ion-exchange column chromatography (Fig. 1) and ion-exchange

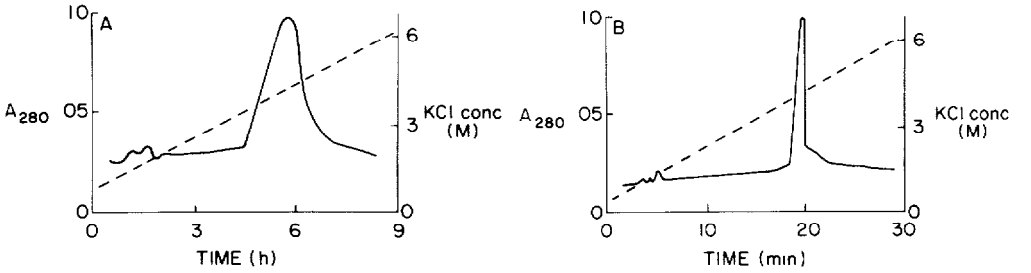

Fig. 1 (left). DEAE Sephadex HPLC of rabbit hind limb myosin extract (500 mg); column, 30 cm × 2.5 cm; flow-rate, 1.0 ml/min; gradient, linear, 500 ml, 0–0.6 *M* potassium chloride; collection time, 9 h.

Fig. 2 (right). Ion-exchange HPLC of rabbit hind lumb myosin extract (100 mg) using the two-pump method; column, DEAE-5PW 7 5 cm × 0 75 cm; flow-rate, 1.0 ml/min at a pressure of 17.2–34.4 Mbar; gradient, 30 min linear, 0–0.6 *M* potassium chloride; collection time, 30 min.

HPLC (Fig. 2). In both cases, a single myosin peak with a pronounced tail was collected when the potassium chloride concentration reached about 0.25 *M*. However, the HPLC method resulted in a sharper peak eluting in approximately 20 min instead of 4 h. A variety of skeletal, cardiac and smooth muscle myosin extracts were successfully separated by ion-exchange HPLC. The results clearly demonstrated that both skeletal and cardiac myosin eluted at approximately 0.25–3.0 *M* potassium chloride while smooth myosin eluted between 0.35 and 0.4 *M* potassium chloride.

Biopsy samples (20–30 mg) obtained from human ventricles were extracted overnight using the procedure outlined in the Experimental section. Unretained material from samples loaded onto a DEAE-5PW column were eluted with 20 m*M* sodium pyrophosphate, pH 8.8. Next, the buffer was changed to 20 m*M* Tris–HCl, pH 7.6, to remove phosphate from the system for ATPase measurements of the HPLC fractions. The results in Fig. 3 clearly show that a small peak of material was collected in the position anticipated for myosin. High-resolution gel analysis confirmed the presence of myosin essentially free from contaminating myofibrillar proteins. Human ventricular myosin was effectively separated from actin and tropomyosin by HPLC as seen in Fig. 4. The higher-molecular-mass band residing above the myosin heavy chain (HC) is thought to be either coalesced myosin HC fragments or filamin. Only slight degradation of the myosin HC was detected, presumably as a consequence of the short analysis time and exposure to alkaline pH.

Small volumes of dilute pregnant rat uterus myosin extract were successfully eluted using a one-pump, step gradient technique. Two discrete peaks were obtained between 0.30 and 0.40 *M* potassium chloride (Fig. 5). The second peak displayed the prominent tail characteristic of myosin. The first peak produced no bands while the second peak displayed relatively purified myosin

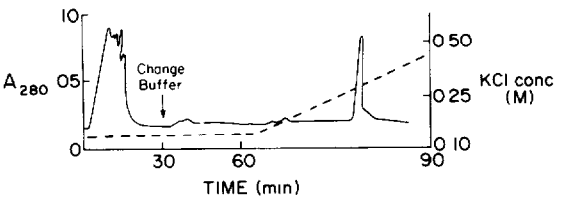

Fig. 3. Ion-exchange HPLC of biopsied human ventricle myosin extract (20–30 mg) using the two-pump method; column, DEAE-5PW 7.5 cm×0.75 cm; flow-rate, 1.0 ml/min at a pressure of 17.2–34.4 Mbar; gradient, 30 min linear, 0–0.5 *M* potassium chloride; collection time, 90 min.

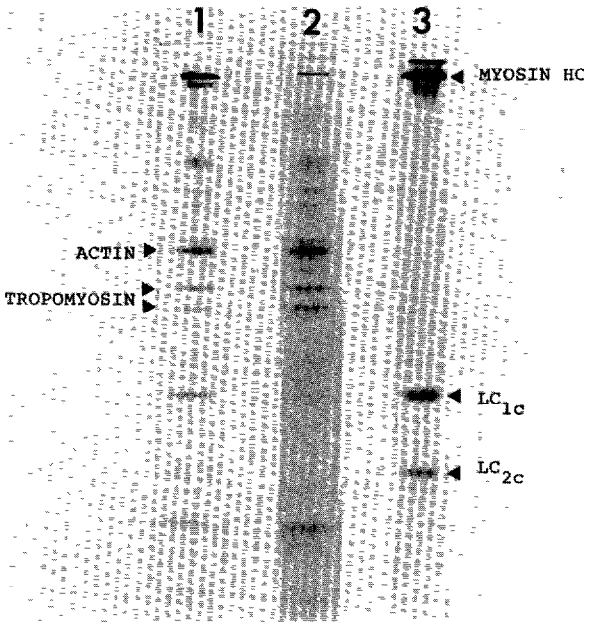

Fig. 4. High-resolution SDS gel electrophoresis of human ventricular myosin fractions from ion-exchange HPLC; Lane 1, biopsied human ventricular myosin extract before HPLC (20 μg); lane 2, sample of unretained material (10 μg); lane 3, sample from protein peak eluted (10 μg) at 0.25 *M* potassium chloride. HC = heavy chain, LC = light chain.

when analyzed by high-resolution SDS gel electrophoresis. Since the 50 m*M* sodium pyrophosphate buffer was not used in this method, the first peak is thought to be ATP which can be also bind to the DEAE matrix.

ATPase activity was recovered in all fractions containing myosin as summarized in Table I. These results demonstrate the feasibility of using a direct extraction technique coupled with ion-exchange HPLC to recover small quantities of biologically active myosin.

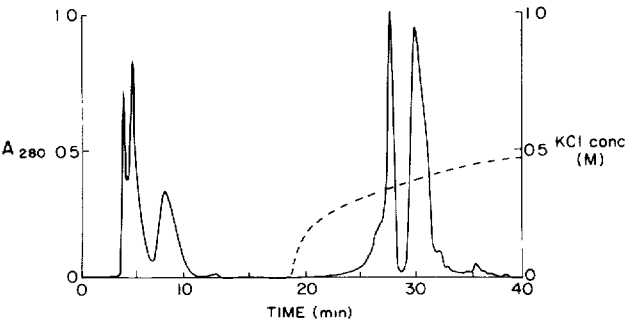

Fig. 5. Ion-exchange HPLC of a thirteen-day-pregnant rat uterus myosin extract (2 mg) using the one-pump method; column, DEAE-5PW; flow-rate, 0.8 ml/min; gradient, step gradient, 0–0.6 *M* potassium chloride; collection time, 35 min.

TABLE I

MYOSIN ATPase ACTIVITY OF COLUMN FRACTIONS OBTAINED FROM A DEAE-5PW HPLC COLUMN

Sample	ATPase activity (μmol inorganic phosphate per mg protein per min)	
	Ca^{2+}-activated	K^+-EDTA-activated
Sample loaded onto the column	0 87	1 05
Myosin peak from a DEAE-Sephadex column	0.76	0.91
Myosin isolated from a biopsy sample using a DEAE-5PW column	0.90	1 06

DISCUSSION

Ion-exchange HPLC can be used for the isolation of myosin on a large scale or from small biopsy specimens. HPLC has the following advantages over conventional open-column chromatography: (a) short analysis times of 20–40 min; (b) a well resolved protein peak eluting between 0.25 and 0.3 *M* potassium chloride for skeletal and cardiac myosin, and between 0.35 and 0.4 *M* potassium chloride for smooth muscle myosin; (c) quantitative recovery of material from the HPLC column avoiding sample dilution.

The rapid protein separation on the column provides a preparation of myosin showing less evidence of degradation as compared to more conventional techniques. These two methodologies are ideally suited to isolate myosin from very small biopsy samples (0.1 g) in sufficient quantities to conduct both biological ATPase activity analysis and gel electrophoresis.

The one-pump method which used less equipment and eliminated loading

the sample with sodium pyrophosphate solution produced results similar to the two-pump technique in regards to both resolution and purity. Subsequent analyses which included using sodium pyrophosphate buffer produced only one elution peak which contained myosin essentially free from myofibrillar contamination.

ACKNOWLEDGEMENTS

Supported by NIH Grant HL 27231 (P.D.A.). Dr. Lema was the recipient of the Parker B. Francis Investigatorship in Anesthesiology 1985–1987.

REFERENCES

1 S.V. Perry, Methods Enzymol., 3 (1955) 582.
2 I. Syrovy, J. Chromatogr., 300 (1984) 225–247.
3 G. Offer, C. Moos and R.J. Starr, J. Mol. Biol., 74 (1973) 653–676.
4 T. Obinata, F.C. Reinach, D.M. Bader, T Masaki and D.A. Fischman, Dev. Biol., 101 (1984) 116–124.
5 F.A. Sreter, S. Holtzer, J. Gergely and H.J. Holtner, J. Cell. Biol., 55 (1972) 586–594.
6 M.J. Lema, E.D. Pagani, R. Shemin and F.J. Julian, Circ. Res., 59 (1986) 115–124.
7 O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.S. Randall, J. Biol. Chem., 193 (1951) 265–275.
8 F.A. Sreter, M. Balint and J. Gergely, Dev. Biol., 46 (1975) 317
9 H.V. Kolbe, D. Costello, A. Wong, R.E. Lu and H. Wohlrad, J. Biol. Chem., 259 (1984) 9115–9120.
10 U.K. Laemmli, Nature, 227 (1970) 680–685.