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Note

Purification of myosin by high-performance liquid chromatography on hydroxyapatite

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Certain proteins and peptides from complex sources seem to possess a great affinity for the support materials commonly used in high-performance liquid chromatography (HPLC), to which they frequently bind irreversibly. The nature of the forces that result in the low recovery of these molecules is often unclear. This is also the case for myosin, the major component of thick filaments in various muscle cells. Myosin consists of two heavy chains (relative molecular mass 200 000) and four light chains relative molecular mass 20 000) assembled together by non-covalent interactions. The existence of myosin isoenzymes that differ in the composition of the light and heavy subunits is now well established not only in skeletal muscle but also in the atrial and ventricular myocardium (see ref. 1 for a review). So far only myosin light chains[2] or their proteolytic fragments[3,4] could be separated by reversed-phase chromatography. Indeed, even using wide pore supports, the purification of the whole myosin molecule by conventional reversed-phase chromatography is difficult.

A new type of column packed with hydroxyapatite has recently been introduced (Bio-Rad Laboratories) which besides unique selectivities, seems to offer valuable advantages over other methods for separating and purifying proteins. The protein–hydroxyapatite interactions are a function of the net charge on the protein, whether acidic or basic[5]. Thus, the fundamental adsorption and desorption mechanisms of hydroxyapatite chromatography resemble those of ion-exchange chromatography, and the selective elution of proteins is achieved by increasing the concentration of the buffer in the mobile phase.

I have made use of this column for purifying the whole myosin molecule, *i.e.*, heavy and light chains, from complex mixtures of proteins under non-denaturing conditions. Evidence is here reported that such a column can be successfully utilized for obtaining a highly purified myosin from those biological tissues for which classical methods appear unsuitable.

EXPERIMENTAL

Reagents

Water was distilled from glass apparatus and filtered through a 0.45-μm Millipore filter. Sodium phosphate buffer, pro analysis grade, was obtained from Merck.

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Myosin preparation

The rabbit muscles analyzed in the present study were the adductor magnus (a fast-contracting muscle), the soleus (a slow-contracting muscle), the ventricular myocardium and the atrial myocardium. Myosin was prepared according to Dalla Libera *et al.*[6] with the modifications reported[2]. Briefly, myosin was isolated from tissue homogenates by means of a high ionic strength buffer containing Mg-ATP. Myosin was then precipitated by dialysis against a low ionic strength buffer and collected by centrifugation. Prior to injection, myosin solubilized in 0.5 *M* potassium chloride–50 m*M* Tris pH 7.6 was precipitated by ten-fold dilution in distilled water. After low speed centrifugation the pellet obtained was resuspended in a small volume of 0.3 *M* sodium phosphate buffer, pH 7.0.

Apparatus

A Perkin-Elmer HPLC system was used as described previously[3]. The hydroxyapatite column (100 mm × 7.8 mm) was obtained from Bio-Rad Laboratories (Bio-Gel HPHT). A guard column (50 mm × 4.0 mm, Bio-Rad Laboratories) filled with an inert, hydrophilic, spherical polymer matrix was always used to protect the main column.

HPLC

All chromatographic experiments were carried out at room temperature at a flow-rate of 0.8–1.0 ml/min. The column was equilibrated with solvent A, consisting of 0.3 *M* sodium phosphate buffer pH 7.0. Myosins were eluted with a 25-min linear gradient to 0.5 *M* sodium phosphate buffer pH 7.0 (solvent B). The effluent was monitored at 280 nm. A 5-min washing step with concentrated phosphate buffer, a 5-min reverse linear gradient and a short reequilibration period were necessary for satisfactory reproducibility.

Electrophoretic analysis

Sodium dodecyl sulphate electrophoresis on polyacrylamide gel slabs was used for testing the degree of purity of original myosin preparations and for analyzing the constituents of the eluates. Gel electrophoresis was carried out according to Laemmli[7], using 15% acrylamide. The gels were stained with Coomassie brilliant blue.

RESULTS AND DISCUSSION

Fig. 1 illustrates typical chromatograms of 100 μg of crude myosin preparations from different rabbit muscles obtained by using HPLC on an hydroxyapatite column and applying a linear molarity gradient of sodium phosphate buffer (pH 7.0). Two main and well separated fractions are recognizable, the first of which (arrows in Fig. 1) is eluted a few minutes after starting. In the case of myosins derived from skeletal muscles (Fig. 1A and B) this fraction is formed by a group of peaks very close to each other. Myosins from ventricular and atrial myocardium display a single peak (Fig. 1C and D). The second fraction is eluted later, about 15 min after the application of the phosphate gradient (stars in Fig. 1). The peak of this fraction is not symmetrical but is broad with tailing at its right-hand side.

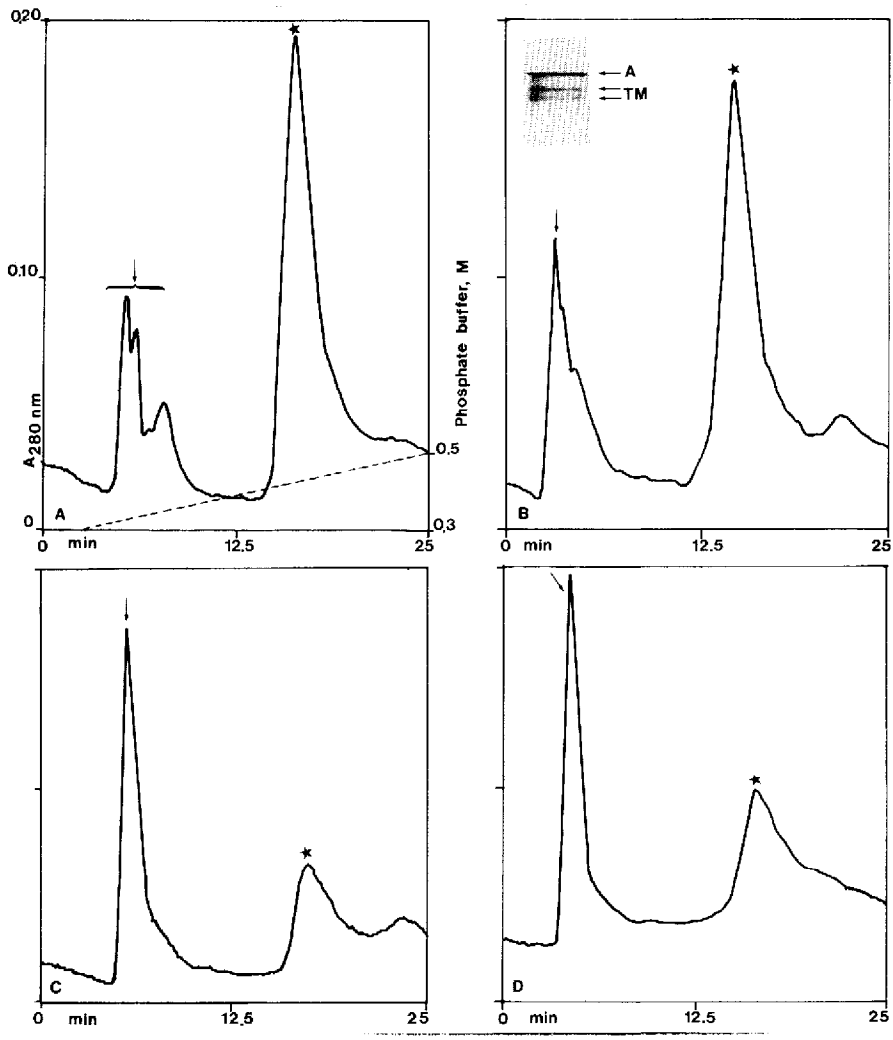

Fig. 1. Chromatograms of myosin from different rabbit muscles by using hydroxyapatite HPLC. About 100 μg of the "crude myosin" preparations were loaded on the hydroxyapatite column. Solvents: A = 0.3 *M* sodium phosphate buffer pH 7.0; B = 0.5 *M* sodium phosphate buffer pH 7.0. Following sample application the proteins were eluted at 1.0 ml/min with a linear gradient from 0 to 100% solvent B over 25 min. The eluate was monitored at 280 nm. Sources of myosin: (A) adductor muscle; (B) soleus muscle; (C) ventricular myocardium; (D) atrial myocardium. The arrows indicate an early eluting group of peaks in each chromatogram, the stars the peaks which contain myosin. Panel B inset: sodium dodecyl sulphate gel electrophoresis on 15% acrylamide of the chromatographic fraction corresponding to the early eluting group of peaks. A = actin; TM = tropomyosin.

The nature of these two fractions was determined by polyacrylamide gel electrophoresis. The first fraction eluted contains actin and tropomyosin (inset in Fig. 1B). As shown in Fig. 2, only myosin subunits, namely myosin heavy and light chains, are present in the second fraction. In fact, actin and tropomyosin, the major contaminants of crude myosin preparations (Fig. 2, lanes a, b and c) are virtually absent in

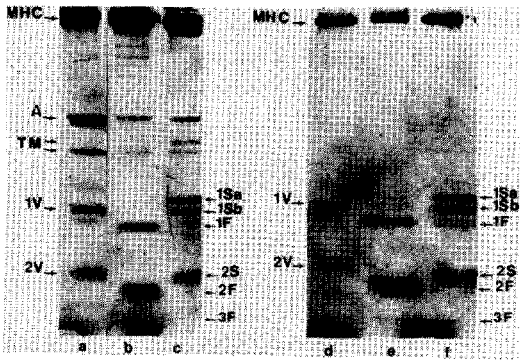

Fig. 2. Sodium dodecyl sulphate gel electrophoretic analysis of rabbit myosins before (a, b, c) and after (d, e, f) hydroxyapatite column purification. Sources of myosin: (a, d) ventricular myocardium; (b, e) adductor muscle; (c, f) soleus muscle. MHC = Myosin heavy chains; A = actin; TM = tropomyosin. 1V, 2V = Light chains characteristic of myosin from ventricular myocardium; 1Sa, 1Sb, 2S = light chains characteristic of myosin from slow-contracting (soleus) muscle; 1F, 2F, 3F = light chains characteristic of myosin from fast-containing (adductor) muscle. The black zones here and there present at the bottom of the figure are photographic artifacts due to the curled edges of the slab.

gel electrophoretic analyses of the second peaks eluted (Fig. 2, lanes d, e and f). Thus, by HPLC on an hydroxyapatite column myosin can easily be purified from different striated muscles, under non-denaturing conditions.

As already mentioned, myosin is a polymorphic molecule the expression of which is not only species- but also tissue-specific. In order to analyze whether or not

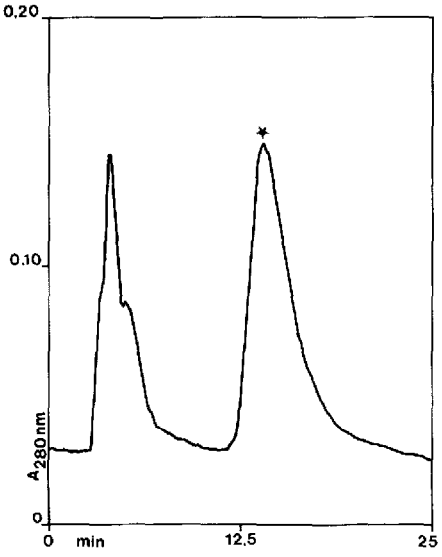

Fig. 3. Chromatogram of a mixture containing about the same amount (50 μg each) of myosin from both adductor and soleus muscles. Experimental conditions as in Fig. 1. Individual chromatograms of adductor and soleus myosin are shown in Fig. 1A and B, respectively. The star indicates the myosin-containing peak.

myosins from different muscles give rise to differences in retention times when chromatographed on hydroxyapatite, a co-analysis of two myosin preparations was performed. Equal amounts of crude myosins obtained from adductor and soleus muscles were mixed and chromatographed. As shown in Fig. 3, myosin was eluted as a single and asymmetric peak, similar to those shown in Fig. 1.

As already shown by electrophoretic analysis, this peak does not contain contaminants, such as actin and tropomyosin. Therefore the asymmetry of the peak could be related to the presence of myosin isoenzymes in the eluate. On the other hand, the single peak obtained in co-analysis experiments of myosin mixtures suggests that by the proposed method we are still unable to separate different myosin isoenzymes. Therefore further experiments are required to find out the optimum separation conditions for the isoenzymes.

It can be concluded that HPLC on an hydroxyapatite column seems to be a good method for (i) highly purifying myosin from samples in submilligram amounts, (ii) under non-denaturing conditions and (iii) in less than 20 min. Finally the procedure can be applied to both skeletal and cardiac muscles. The method is particularly advantageous when classical methods for myosin extraction are unsuitable to obtain the degree of purity necessary for enzymatic and structural studies.

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