A NEW AND RAPID METHOD FOR THE ISOLATION OF MYOSIN FROM SMALL AMOUNTS OF MUSCLE AND NON-MUSCLE TISSUE BY AFFINITY CHROMATOGRAPHY

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1. Introduction

The immobilization of adenosine nucleotide derivatives [1] has proved to be a general affinity chromatographic medium which can be used for a variety of enzymes. Insolubilized AMP derivatives have been used extensively both to purify and study the mode of action of dehydrogenase [1–4] whereas agarose conjugates of ADP and ATP derivatives have proved excellent media for the chromatography of kinases [1,5] and ATPases [1,6–8].

The contractile protein, myosin, with its requirement to interact reversibly with both actin and adenosine nucleotides during muscle contraction, represents an ideal case in which to exploit this technique. Conventional methods for purifying this protein are based upon the fact that large quantities of starting muscle tissue are usually available and they are not easily adaptable to the situation where either the supply of muscle is limiting or myosin is to be prepared from other sources. In this report we describe a method of purifying myosin direct from tissue extracts by affinity chromatography on immobilized ADP derivatives which can be readily applied to isolating this protein from very small quantities of muscle tissue or from non-muscle sources.

2. Materials and methods

2.1. *Preparation of muscle extracts*

Minced rabbit muscle (1 g) was extracted for 30 min at 4°C with a 5 volume excess of 0.6 M ammonium acetate, 2 mM magnesium acetate, 2 mM sodium pyrophosphate, 0.5 mM dithiothreitol, pH 6.5. The extract was spun at 2000 *g* for 15 min and the supernatant filtered through glass wool. The filtrate was diluted about 5-fold with column buffer: 0.6 M ammonium acetate, 5 mM EDTA, 2 mM sodium pyrophosphate, 0.25 mM dithiothreitol, pH 7.5 (Buffer A). The resulting solution was applied directly to the affinity column equilibrated in Buffer A.

2.2. *Preparation of pig platelet extracts*

Frozen/thawed platelets (1 g wet packed weight) were extracted as above except that a 10-fold excess of buffer was used. The extract was spun at 80 000 *g* for 15 min and the supernatant diluted with an equal volume of water and a further 3 to 5 volumes of 0.3 M ammonium acetate containing 2 mM magnesium acetate, 2 mM sodium pyrophosphate and 0.25 mM dithiothreitol, adjusted to pH 7.5 with 5 M ammonium hydroxide, was added (Buffer B). This solution was applied directly to the affinity column equilibrated to Buffer B.

2.3. *Affinity columns*

8-(6-Aminohexyl) amino-ADP (C8-ADP) and N^6-(6-aminohexyl)-ADP (N^6-ADP) were prepared as described earlier [1]. These ligands were coupled to CNBr-activated Sepharose-4B essentially as described by Axen et al. [9] under the conditions given by Trayer et al. [1]. Ligand concentrations of between 0.5 and 5 μmol nucleotide per g wet packed gel have been successfully employed.

2.4. *Chromatographic procedures*

All affinity columns were operated at 4°C at a flow rate of 15–20 ml/hr. Between runs the columns were washed with a mixture of 2 M KCl and 6 M urea,

followed by the starting buffer. Detection of protein eluting with ATP was determined by the turbidimetric microtannin method of Mejbaum-Katzenellenbogen and Dobryszycka [10] using as a standard myosin prepared from the longissimus dorsi muscle of New Zealand White rabbits [11]. Light scattering was measured at 500 nm in this procedure.

2.5. *Enzyme assays*

ATPase activities of myosin were carried out by direct determination of P_i [12]. Incubations [11] were carried out in a final volume of 1.0 ml under the following conditions: Ca-ATPase: 5 mM calcium acetate, 5 mM ATP, 30 mM ammonium acetate and 50 mM Tris–HCl, pH 7.5; NH_4-ATPase: 5 mM ATP, 2 mM EDTA, 0.6 M ammonium acetate, pH 7.5. Muscle myosins were incubated at 25°C and platelet myosin at 37°C.

2.6. *Polyacrylamide gel electrophoresis*

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate was carried out by the method of Weber and Osborn [13] using gels containing 8% acrylamide. The stained gels were scanned on a Gilford 2000 recording spectrophotometer, fitted with a model 2410 gel scanner. Polyacrylamide gels (8%) in 8 M urea, 20 mM Tris–125 mM glycine, pH 8.6, were performed according to the method of Perrie and Perry [14].

3. Results and discussion

3.1. *Rabbit muscle*

Preliminary experiments in which rabbit muscle myosin, purified by conventional techniques, was applied directly to Sepharose derivatives of either C8-ADP or N^6-ADP indicated: (a) that this enzyme could be bound quite tightly to these columns in the presence of either Mg^{2+}, Ca^{2+} or in the absence of divalent cations (in the presence of EDTA) at the high ionic strength required for its solubility; and (b) that buffers containing high concentrations of Cl^- inhibited this binding [7]. The final buffer chosen for the chromatography work therefore contained 0.6 M potassium or ammonium acetate in order to solubilize the myosin. Binding of myosin to these Sepharose-ADP derivatives in the presence of Mg^{2+} or Ca^{2+} or in the presence of EDTA in the K^+ or NH_4^+ buffers represents the three conditions under which myosin ATPase activity can be expressed in vitro.

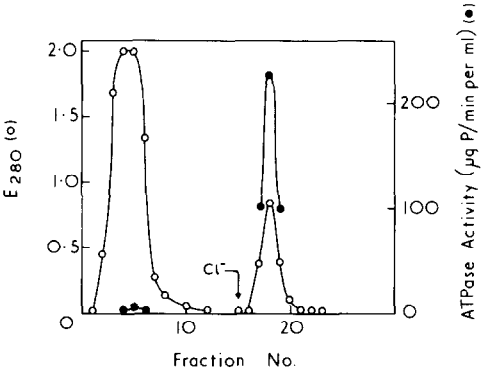

Fig.1. Chromatography of an extract of rabbit longissimus dorsi muscle (1 g) on a Sepharose-N^6-ADP column 8 cm long and 0.8 cm diameter. Conditions for extraction of the muscle and operation of the column are given in Materials and methods. At the arrow, 0.6 M ammonium chloride replaced the ammonium acetate in the developing buffer.

It was decided initially to bind selectively myosin direct from muscle extracts by operating the columns in the presence of EDTA since most other ADP-binding proteins likely to be found in muscle extracts would require a divalent cation for expression of this activity. Later experimentation, however, showed that identical results could be obtained if Mg^{2+} (5 mM) were included in these buffers and EDTA omitted. Fig.1 describes what happens when an extract from 1 g rabbit longissimus dorsi was applied to an agarose-N^6-ADP conjugate. After washing the column until the E_{280} of the eluate had dropped to zero, a protein peak could be eluted either by replacing the ammonium acetate with 0.6 M ammonium chloride, or by including 50 mM ATP in Buffer A. The purity of the myosin prepared in this manner can be seen after subjecting the chloride eluate to polyacrylamide electrophoresis in the presence of sodium dodecyl sulphate (SDS) fig.2(c). In fig.2(d) the trace of an SDS-gel of myosin prepared by conventional procedures [11] is shown for comparison. The myosin prepared by affinity chromatography is indistinguishable from 'standard' myosin preparations and its enzymic activities are invariably higher, having specific ATPase activities (in μmol P/min/mg) of 1.2 and 5 in the presence of Ca^{2+} and NH_4^+ (+ EDTA)

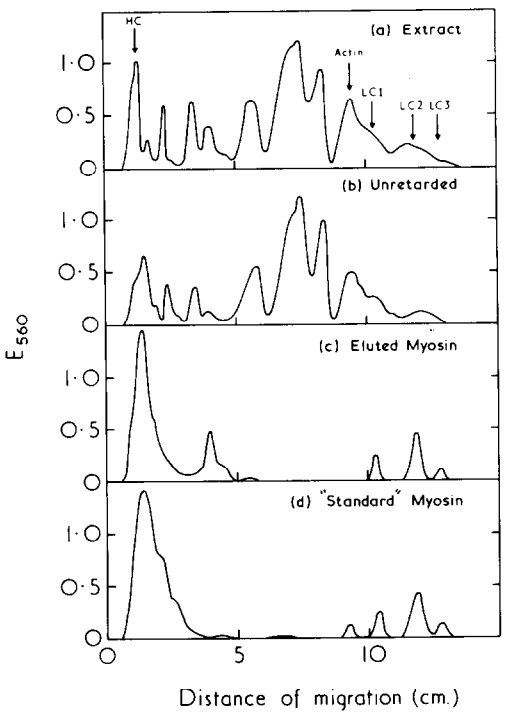

Fig.2. Absorbance profiles of SDS-polyacrylamide gel electrophoresis of rabbit myosin fractions prepared as shown in fig.1. (a) Muscle extract applied to column, (b) inactive material appearing in the unretarded fraction, (c) material eluted by Cl^- or ATP and (d) sample of myosin prepared by conventional procedures [11]. The position of migration of the myosin heavy chains (HC) and light chains (LC 1,2 and 3) and actin are shown.

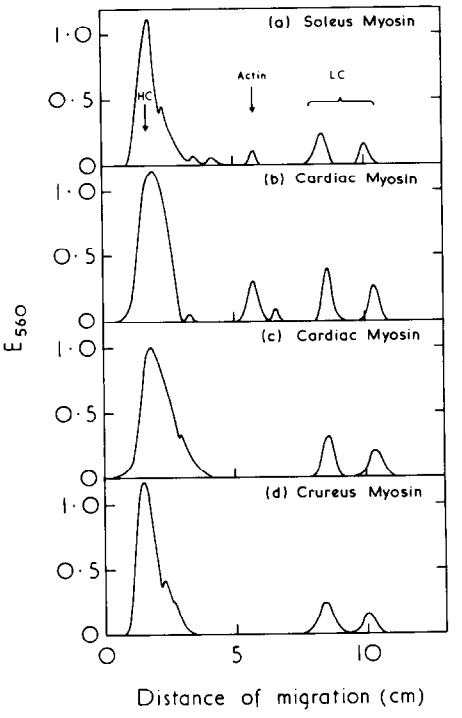

Fig.3. Absorbance profiles of SDS-polyacrylamide gel electrophoresis of myosins prepared from different rabbit muscle by affinity chromatography on Sepharose-N^6-ADP. Each preparation was made from about 1 g of muscle as described in Materials and methods. Profiles (a) and (b) were made on myosins eluted directly from the affinity columns, whereas in (c) and (d) the myosins were also subjected to gel filtration [15]. Only two light chains are seen in (a) and (d) since 8% polyacrylamide gels do not resolve the two heavier light chains of red muscle myosins [16].

respectively. The Mg·ATPase activity was readily stimulated by actin. This method quantitatively removes the enzymically-active myosin from the applied extracts and some inactive myosin does appear in the unretarded fraction. Yields of between 10–20 mg myosin per g muscle are obtained. No effort has yet been made to increase the amount of myosin in the initial extract.

This method was also applicable to the isolation of myosin from cardiac muscle and from the red, slower-contracting, soleus and crureus muscle of rabbit. The myosins so isolated again possessed all the biological activities found in such myosins prepared by conventional procedures. The purity of the myosins prepared from these muscle by affinity chromatography can be seen in fig.3. In these experiments the myosin was eluted from the affinity columns stepwise by the addition of 50 mM ATP to Buffer A. Occasionally small amounts of actin and some heavier impurities were found in these preparations (figs.3a and b) but these were readily removed by gel filtration on Sepharose-4B in the presence of 0.6 M KCl and 0.5 mM ATP [15]. This method could be readily scaled down to handle small amounts of material and resulted in very pure myosin preparations (figs.3c and d). Elution by an ATP gradient rather than stepwise elution also improved the purity of these myosin preparations. Two protein peaks emerged from the column; the first, eluting at the lower ATP concentration, was myosin and the second contained mainly

actin. This would imply that the actin was binding directly to the immobilized ADP derivative rather than to the myosin–ADP complex.

3.2. *Blood platelet myosin*

A summary of the results obtained for pig platelet myosin can be seen in fig.4. All the ATPase activity

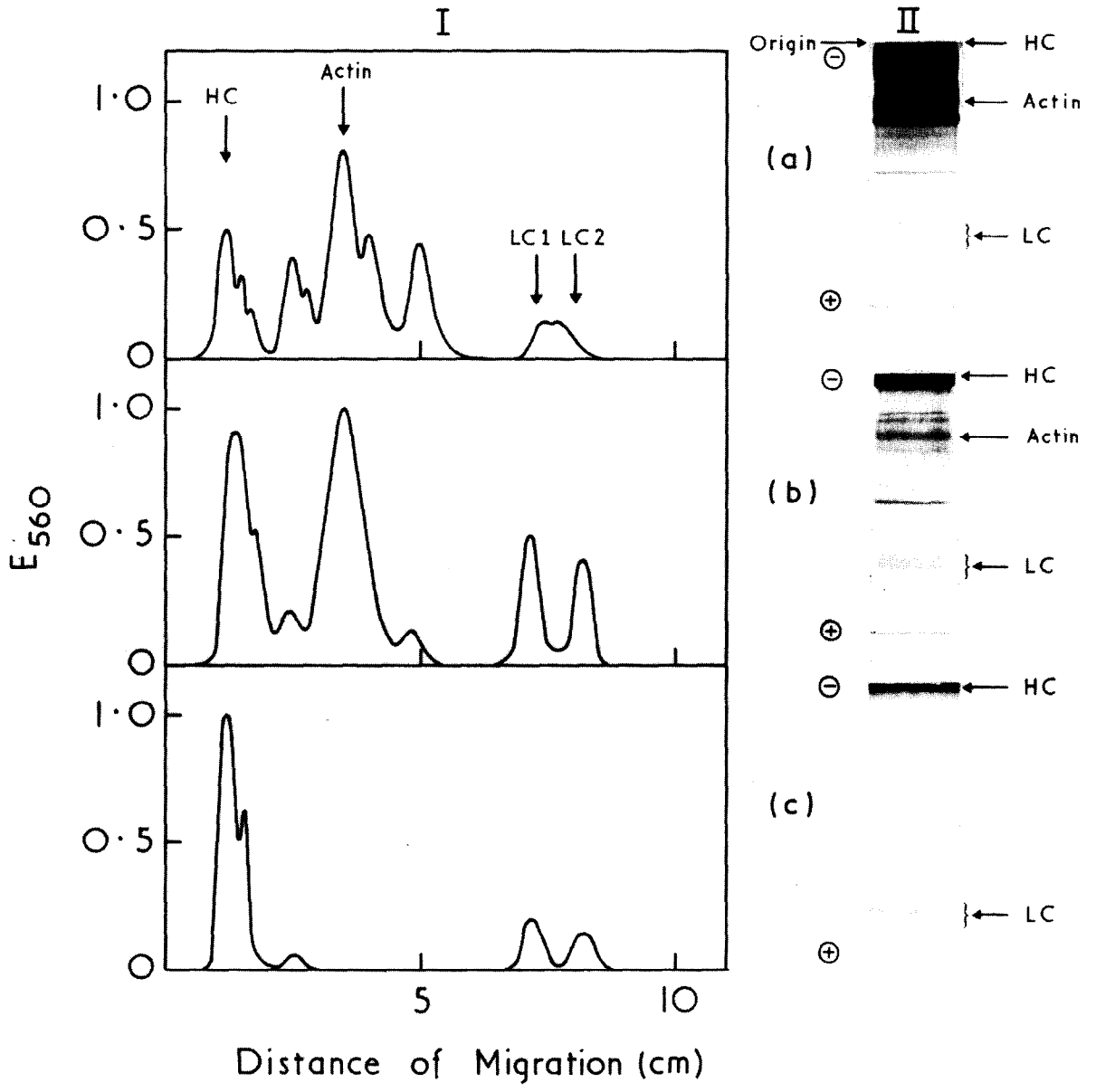

Fig.4. Polyacrylamide gel electrophoresis of pig platelet myosin preparations I, Absorbance profiles of SDS-polyacrylamide gel electrophoresis; II, polyacrylamide gel electrophoresis in the presence of 8 M urea, pH 8.6 [14]. (a) Platelet extract applied to Sepharose-N^6-ADP, (b) actomyosin eluted from Sepharose-N^6-ADP by 50 mM ATP, and (c) myosin purified further by gel filtration on Sepharose-4B. The position of the myosin heavy chains (HC) and light chains (LC 1, LC 2) and actin are indicated. After dialysis against 0.6 M ammonium acetate 0.25 mM dithiothreitol, pH 7.5, the specific ATPase activities (μmol P/min/mg) of the various stages of the preparation were (a) 0.1 (NH_4^+/EDTA) (b) 0.3 (NH_4^+/EDTA) and (c) 2.5 (NH_4^+/EDTA) and 0.8 (Ca^{2+}).

found in the platelet extract was present in the supernatant after removal of the insoluble material by centrifugation. Re-extraction of this pellet did not solubilize any additional ATPase activity. All of the ATPase activity in the extract supernatant was retained on the affinity columns when the ammonium acetate concentration in the column buffers was reduced to 0.3 M. Platelet myosin could also be bound to the agarose-N^6-ADP in the presence of either EDTA or Ca^{2+} ions, but in this instance binding appeared to be a little more efficient in the presence of Mg^{2+} ions; thus the columns were operated in Buffer B. The myosin was specifically eluted from these columns by including 50 mM ATP in this buffer. The resulting material (fig.4b) still contained a considerable amount of actin but this could easily be removed by gel filtration on Sepharose-4B using the discontinuous buffer system of Pollard et al. [15]. Using this method, 1 mg of purified platelet myosin can be obtained from 1 g of platelets, possessing specific ATPase activities higher than any others reported in the literature (fig.4). It was interesting to note that if the affinity columns were eluted by an ATP gradient, then again two protein peaks were detected. In this case, the peak eluting at the lower ATP concentration (10 mM ATP) contained mostly actin (> 70%) whereas the second peak (25 mM ATP) contained chiefly myosin although some actin was still present.

4. Concluding remarks

The method described here can be applied to the purification of myosin from very small amounts of tissue – the detection of the eluting myosin being the only consideration. We have used it successfully with 100 mg of muscle tissue or blood platelets and there is no reason why smaller amounts of tissue cannot be used. This means that myosin should be easily obtained in an afternoon's work (overnight if the Sepharose-4B column is used) from, for example, muscle tissue culture samples, biopsy samples and a variety of non-muscle tissues where at the moment conventional procedures are not easily applicable. Yields are virtually quantitative from the extracts and improvement of the extraction procedures would mean that even smaller amounts of tissue could be used.

We have noticed during our gradient elution procedures that the myosin from different muscle types emerge from the columns at different but distinctive ATP concentrations (all other factors being kept constant). This elution position appears to be related to the specific ATPase activity of the individual myosin-type. It was also noted that some of these myosins from single muscle types eluted under the ATP gradients as broad peaks. This may be due to either the fact that the myosin molecule is highly asymmetrical and steric factors may influence its interaction with the immobilized ADP or an heterogeneity of the myosin molecules themselves if they coexist as isoenzymes in a single tissue. We are currently investigating this possibility.

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