A SPECIFIC METHOD FOR THE PREPARATION OF PURE MYOSIN

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1. Introduction

It has been clearly shown by electrophoresis on SDS-polyacrylamide gels [1, 2] that myosin prepared in the usual manner, as described initially by S.V. Perry [3] and considered until lately as pure, contains small amounts of several other proteins of intermediate molecular weight. Inasmuch as many of these are of myofibrillar origin and firmly bound to myosin, they could not all be considered as impurities in the classical meaning [1, 4]. The localization of some of these proteins in the myofilament has been suggested by recent experiments [5, 6]. However their physiological roles are not yet known, although it does seem possible that they have structural significance or influence in some way actin–myosin or myosin–myosin interactions during muscle contraction [5–10].

The purification of myosin and the isolation of its accompanying proteins has been attempted by different workers using chiefly column chromatography with DEAE-cellulose or DEAE-Sephadex [1, 11–13] and ammonium sulphate fractionation [1, 14]. Unfortunately the results obtained by chromatography do not always agree, and in our hands the ammonium sulphate fractionation did not come out successfully. In any case even the best methods do not seem to produce entirely pure myosin, small quantities of components of 160 000–180 000 daltons being always present [1, 2].

Our purpose here was to check or eventually to improve, the chromatographic procedures already published and to look for new methods. As such we used specific interaction with actin and chromatography on Sepharose columns. The progress of the purifications was followed by electrophoresis on SDS polyacrylamide gels.

Abbreviations:

SDS: sodium dodecyl sulphate; DEAE: diethyl amino ethyl.

2. Materials and methods

2.1. *Crude myosin preparations*

Rabbit skeletal myosin was prepared according to the method for S.V. Perry [3], the number of reprecipitations being however reduced to two, since no further purification could be observed by gel electrophoresis beyond this limit. Myosin preparations enriched in actin were obtained by lengthening the initial extraction time from 10 to 45 min and by omitting the intermediate precipitations at 0.30 M KCl, all other conditions being identical to those of the original method.

The stock solutions contained 10 $mg \cdot ml^{-1}$ protein, 0.45 M KCl, 0.02 M potassium orthophosphate, the pH being adjusted to 6.8. They were stored at 4°C and used within one week, except when the influence of the ageing of the proteins was to be studied.

2.2. *Chromatographic procedures*

Two supports were used: DEAE-Sephadex A-50 and Sepharose 2B, both manufactured by Pharmacia, Sweden. The columns were 45 cm long and 2.5 cm in diameter. Sample of 5–15 ml containing 50–150 mg protein were consistently applied. The elutions were conducted upward at a constant rate of 20 $ml \cdot hr^{-1}$. The temperature was always maintained at ±4°C.

When DEAE-Sephadex A-50 was used the technique described by E.V. Richard et al. [13] was followed. The initial solvents were 0.15 M potassium orthophosphate *or* 0.04 M sodium pyrophosphate with 0.01 M EDTA present everywhere and the pH being always adjusted to 7.5. The elutions were realized by KCl

gradients (all other constituents being maintained constant), the slopes of which were varied in the course of the experimentation; the best resolution being obtained with the contours indicated on the figures.

The purpose of the Sepharose 2B chromatographics was double: i) Simply to try to separate myosin from its accompanying proteins. ii) To separate myosin from actomyosin in an enriched preparation to check whether the other proteins followed the myosin or the actomyosin fractions or both. In all cases the solvent contained 0.45 M KCl, 0.01 potassium orthophosphate and 0.005 M $MgCl_2$, the pH being always adjusted to 6.8. In the first case only 0.005 M sodium pyrophosphate was added to dissociate the actomyosin complex.

2.3. *Polyacrylamide gel electrophoresis*

All solutions to be analysed were boiled for 5 min in the presence of 1% SDS and 1% mercaptoethanol to assure proper dissociation and to prevent degradation by proteolytic enzymes [15]. The experimental procedure was that described by U. Lammli [16]. When myosin was tested for purity a minimum loading of 10 μg protein was used. The molecular weights were estimated following the method of K. Weber and M. Osborn [17] with the following as markers, molecular weight in daltons in parentheses: cytochrome *c* (13 400) carboxypeptidase (34 000), actin (46 000), pyruvate-kinase (57 000), bovine serum albumin (68 000), RNA-polymerase from *E. coli* (165 000, 155 000 and 41 000) and the σ-factor of RNA-polymerase (86 000).

3. Results

3.1. *Crude myosin preparations*

Our preparations of myosin purified according to the techniques of Perry [3] contain essentially the same constituents as those of Starr and Offer [1], the apparent molecular weights being nevertheless somewhat different. Within the limits of resolution of the electrophoresis techniques used, the preparations enriched in actin do not contain any supplementary proteins, if one excepts the actin itself (fig. 1).

3.2. *Chromatography on DEAE-Sephadex A-50*

We found that when orthophosphate *or* pyrophosphate were present the separations are not the same, which is at variance with previous statements [1, 13]. In the presence of pyrophosphate (fig. 2a) we did obtain the same results as Starr and Offer [1]; a clear separation of two components of respectively 90 000 and 150 000 daltons from the remainder. In the presence of orthophosphate none of these proteins were separated entirely from the remainder and the 150 000 daltons component was remarkably concentrated in the trailing end of the myosin peak (fig. 2b). The origin of this difference was not clear; our results being very reproducible. In aged preparations (more than two weeks old) myosin appeared in the first elution peak, which is in harmony with the statements of Richard et al. [13], who introduced the techniques to

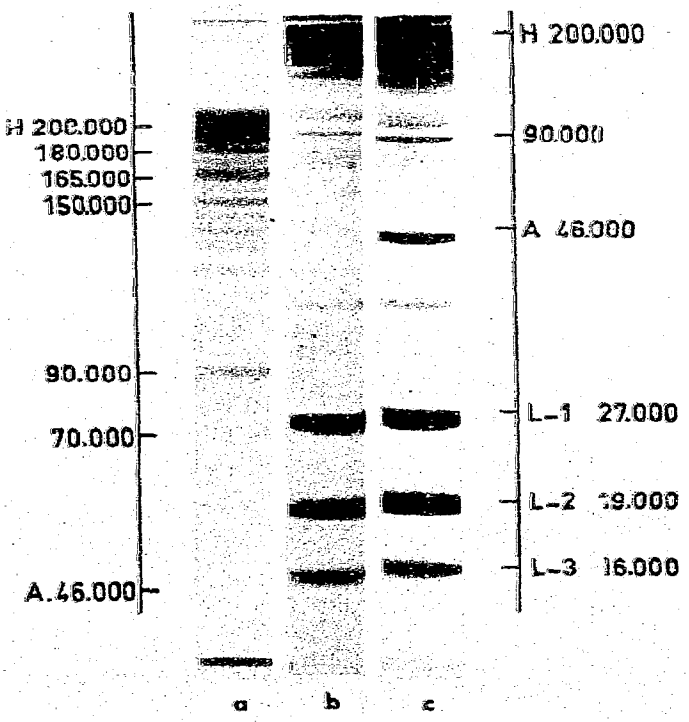

Fig. 1. Electrophoresis on SDS-polyacrylamide gels of crude myosin preparations purified according to the technique of S.V. Perry [3] (a and b) and myosin preparations enriched in actin (c). H, L-1, L-2, L-3 stand, respectively, for the heavy and light polypeptide chains which compose the intact myosin molecule dissociated by the SDS and A for actin [18], the other chains corresponding to different proteins. Chain weights obtained by the procedure of Weber and Osborn [17]. Gel concentrations were respectively 7% (a) and 12.5% (b and c).

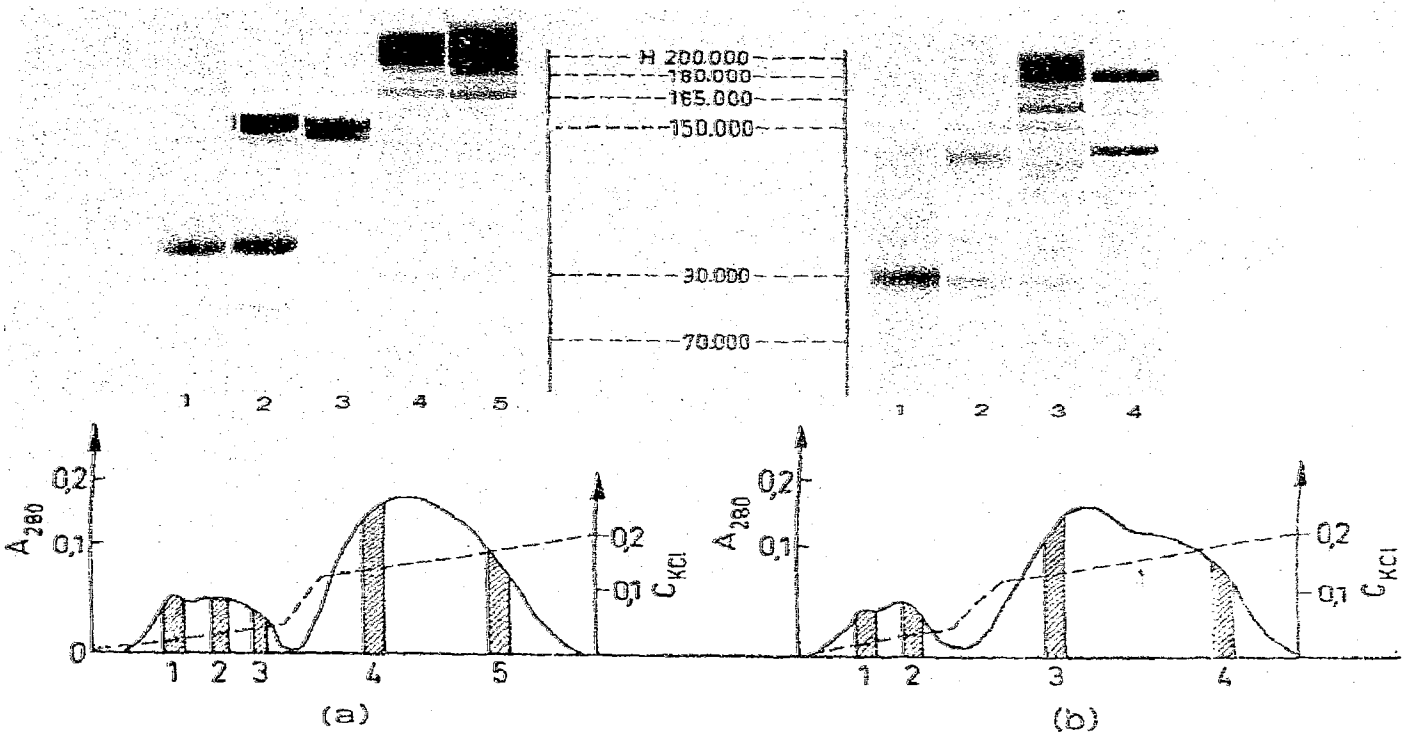

Fig. 2. Elution profiles of myosin purified according to the technique of S.V. Perry [3] chromatographed on DEAE-Sephadex. The hatched zones correspond to the fractions analysed by electrophoresis on 7% SDS-polyacrylamide gels and the dotted lines to the elution KCl gradients. H stands for the heavy myosin chain: a) Initial solvent: 0.04 M sodium pyrophosphate, 0.01 M EDTA, pH 7.5. b) Initial solvent: 0.15 M potassium orthophosphate, 0.01 M EDTA, pH 7.5.

separate intact and altered myosin. In no case the components of 165 000 and 180 000 daltons were separted from the myosin. An extra result, with respect to those obtained by Starr and Offer, was that the use of a very faint KCl gradient makes possible the separation of the components of 90 000 and 150 000 daltons from each other without the help of ammonium sulphate fractionation.

3.3. *Chromatography on Sepharose 2B*

When the actomyosin complex is dissociated byy pyrophosphate only the actin is distinctly separated from the remainder. However, the myosin of the leading front of the major elution peak is relatively cleared of the 150 000 daltons component, this latter being concentrated in its trailing end (fig. 3a).

The situation is quite different when pyrophosphate is absent, and as a consequence, the actomyosin com plex is not dissociated. In this case the actomyosin fraction, clearly separated from the remainder, is practically pure, while the fractions corresponding to free myosin contain almost all the constituents of crude myosin preparations (fig. 3G). Myosin exempt from actin can then easily be obtained by dissociating the actomyosin complex with pyrophosphate or ATP and by submitting the whole to a second passage on Sepharose 2B (fig. 4).

Under these circumstances it appeared to us that pure myosin should be as easily obtained by simple precipitation of actomyosin at concentrations of salt when free myosin remains in solution. In our hands this experiment was not successful, inasmuch as considerable amounts of free myosin, with all its accompanying proteins, coprecipitate with the actomyosin.

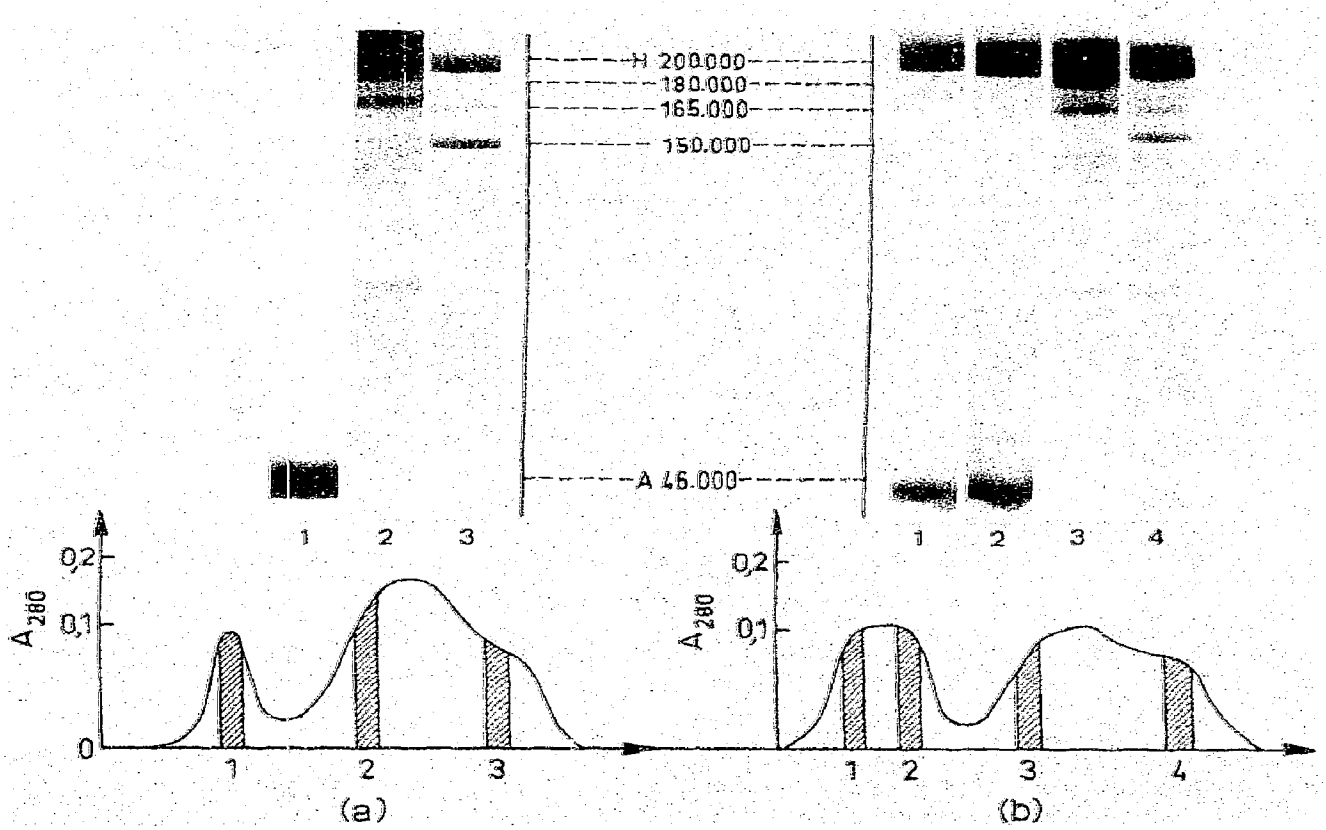

Fig. 3. Elution profiles of myosin preparations enriched in actin chromatographed on Sepharose 2B. The hatched zones correspond to the fractions analysed by electrophoresis on 7% SDS-polyacryiamide gels. H stands for the heavy myosin chain and A for actin. a) Solvent: 0.45 M KCl, 0.01 M potassium orthophosphate, 0.005 M sodium pyrophosphate, 0.005 M $MgCl_2$, pH 6.8. b) Solvent: 0.45 M KCl, 0.01 potassium orthophosphate, 0.005 M $MgCl_2$, pH 6.8.

4. Discussion

To conclude this seems to be, as far as we are aware, the first time that myosin freed from its accompanying proteins of intermediate molecular weight (at least as can be judged from electrophoresis on SDS polyacrylamide gels) has been prepared. The fact that this can be realized by specific interaction within actin seems to indicate that the binding of myosin to actin in the actomyosin complex implies some loosening of the links between myosin and its accompanying proteins and in particular with the components of 165 000 and 180 000 daltons. It is tempting to propose that this has some significance in relation to the events occurring on muscle contraction. Nevertheless it should be kept in mind that as long as we are not able to confer definite physiological roles to these proteins, they could equally well represent degradation products of myosin itself having physical and chemical characteristics close to those of myosin, but devoid of the specific property to interact with actin. Further work should thus explicitly tend to ascertain these eventual physiological functions.

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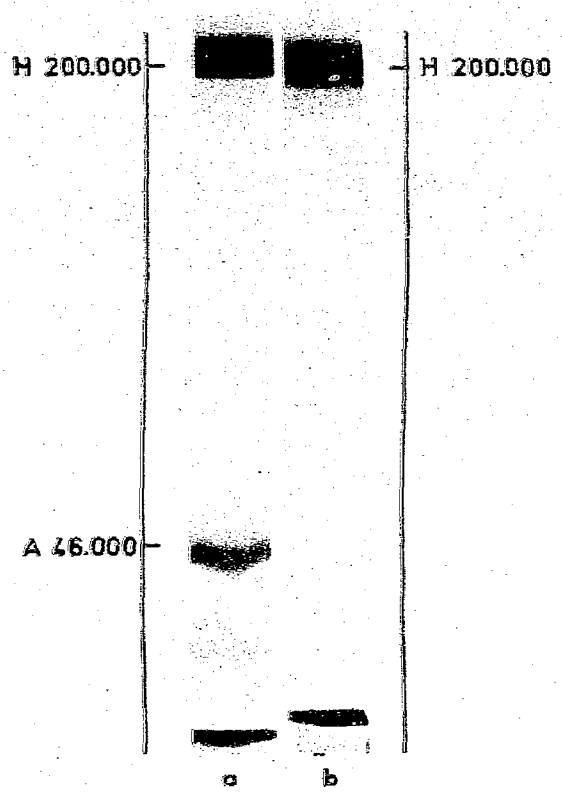

Fig. 4. Electrophoresis on 7% SDS-polyacrylamide gels of actomyosin purified on Sepharose 2B (a), and of myosin obtained by a second passage on Sepharose 2B after the dissociation of the actomyosin complex by sodium pyrophosphate in the presence of $MgCl_2$ (b). H stands for the heavy myosin chain and A for actin.

References

[1] Starr, R. and Offer, G. (1971) FEBS Letters 15, 40.
[2] Lowey, S. and Risby, D. (1971) Nature 234, 81.
[3] Perry, S.V. (1955) in: Methods in Enzymology, Vol. II, p. 582 (Colowick, S.P. and Kaplan, N.O., eds.) Acad. Press, N.Y.
[4] Sender, M.P. (1971) FEBS Letters 17, 106.
[5] Hanson, J. (1972) Abs. IV Int. Congr. Of Biophys. Moscow.
[6] Offer, G. (1972) Abs. XXXVII Cold Spring Harbour Symp. Quant. Biol.
[7] Huxley, H.E. and Brown, W. (1967) J. Mol. Biol. 30, 383.
[8] Masaki, T., Takaiti, O. and Ebashi, S. (1968) J. Biochem. 64, 909.
[9] Moromoto, K. and Harrington, W.F. (1972) J. Biol. Chem. 247, 3052.
[10] Eaton, B.L. and Pepe, F.A. (1972) J. Cell. Biol. 55, 681.
[11] Dubuisson, M. (1946) Experientia 2, 412.
[12] Perry, S.V. (1960) Biochem. J. 74, 94.
[13] Richard, E.F., Chung, C.S., Menzel, D.B. and Olcott, H.S. (1967) Biochemistry 6, 258.
[14] Tsao, T.S. (1953) Biochim. Biophys. Acta 11, 368.
[15] Pringle, J.T. (1970) Biochem. Biophys. Res. Commun. 39, 46.
[16] Lämmli, U. (1970) Nature 227, 680.
[17] Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406.
[18] Weeds, A.G. and S. Lowey (1971) J. Mol. Biol. 61, 701.