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FURTHER OBSERVATIONS ON THE CHROMATOGRAPHY OF MYOSIN PREPARATIONS ON HYDROXYAPATITE

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SUMMARY

An improved procedure for a high ionic strength chromatographic fractionation of myosin preparations on hydroxyapatite was developed and applied to crude preparations of myosin made from fresh and aged bovine and rabbit muscles, and to a preparation containing both sarcoplasmic and myofibrillar proteins made from fresh rabbit muscle. The efficiency of the procedure in removing actin and other contaminating proteins from myosin was demonstrated. The results also indicate that the method would be useful in a study of the compositional changes in muscle on aging.

INTRODUCTION

Hydroxyapatite is a very convenient and useful medium for the chromatographic fractionation of preparations of myosin[1]. Variation in the chromatographic behavior of such preparations with the age of the muscle from which the preparation was made was tentatively attributed to varying degrees of myosin aggregation[1]. This paper reports on a refinement of the fractionation procedure. The improved procedure has been applied to myosin preparations made from fresh rabbit and bovine *Longissimus dorsi* muscles and from rabbit and bovine muscles stored at 4° and 0°C, respectively. The chromatographic fractions have been examined for their protein composition, including actin content, by disc gel electrophoresis. The new procedure has also been applied to an aqueous salt extract of fresh rabbit muscle containing both the sarcoplasmic and myofibrillar proteins. The chromatographic fractions obtained were examined for their content of five selected enzymatic activities, *viz.*, creatine phosphokinase, pyruvate kinase, AMP deaminase, fructose diphosphate (FDP) aldolase, and ATPase. The results are reported and their significance discussed in terms of (1) the efficiency of the improved procedure in removing contaminating proteins from myosin and (2) the change occurring in aging muscle to which much of the variation in chromatographic behavior can be attributed.

Various other chromatographic procedures have been utilized for the puri-

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fication and analysis of myosin preparations. Molecular sieve chromatography on agarose has been proposed for removal of "aggregated myosin and heterogenous low-molecular-weight contaminants"[2], the cation exchanger cellulose phosphate has been used for removal of deaminase[3] and the anion exchangers DEAE-cellulose and DEAE-Sephadex for removal of nucleic acid and aggregated myosin, as well as other contaminants[3,4]. More recently, the highly selective technique of affinity chromatography on immobilized actin and immobilized nucleotides such as ADP and ATP has been introduced[5–7]. The merits of hydroxyapatite relative to the other support media mentioned will be discussed briefly.

MATERIALS AND METHODS*

Protein preparations

Crude myosin preparations were made from *Longissimus dorsi* muscles from steers 18–24 months old and from non-pregnant female albino rabbits 1.6–2.1 kg in weight. The muscles were removed from the animals within 10 min after death. A sample of the fresh muscle was taken and the preparation of myosin was begun immediately. The remainder of the muscle was enclosed in Saran wrap and stored. Bovine muscle was stored at 0°C and rabbit muscle at 4°C. The protein preparations were made, from both fresh and aged muscle, as previously described[8], with the following modifications: (1) β-mercaptoethanol was present at a concentration of 10 m*M* in all reagents used in making the protein preparation, (2) treatment with ribonuclease was not used and (3) the third cycle of precipitation and redissolution was omitted. The muscle was ground with a Hamilton Beach grinder precooled on ice through a grinding plate with 3/16-in. holes. The ground muscle was extracted with Guba–Straub solution for 20 min.

A soluble protein preparation containing both sarcoplasmic and myofibrillar proteins was made from fresh rabbit *Longissimus dorsi* muscle as follows. A 110-g sample of the muscle was ground as described above and extracted with 165 ml Guba–Straub solution[8]. After addition of 200 ml eluent A_1 (Table I), the preparation

TABLE I

COMPOSITION OF ELUENTS

Eluent	*Concentration of component*				*pH at 5°C*
	KCl (M)	*Potassium phosphates (M)*	*NaOH (M)*	*β-Mercaptoethanol (mM)*	
A_1	0.40	0.050	0	10.0	6.8
A_2	0.40	0.16	0	10.0	6.8
B_1	0.40	0.20	0	10.0	6.8
B_2	0.40	0.23	0	10.0	6.8
C	0.40	0.40	0	10.0	6.8
D	0	0	0.10	0	—

* Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

was filtered through cheesecloth, clarified by centrifugation at 3300 *g* for 30 min, dialyzed for 20 h against eluent A_1 and clarified again by centrifugation at 13,200 *g* for 1 h. A 3-ml aliquot of the clarified solution, containing approximately 22.5 g protein/l, was diluted to 10 ml with eluent A_1, and an 8-ml aliquot of the diluted solution, containing 54 mg protein, was applied to a column of hydroxyapatite. Chromatography was as described below. All reagents used in this preparation, as well as eluents A_1–C, contained 1 m*M* dithiothreitol in place of mercaptoethanol. All operations were at 5°C.

Chromatography

Hydroxyapatite available from various manufacturers contained large and probably variable amounts of brushite and was not suitable for the chromatography of preparations of myosin without further treatment. To obtain a satisfactory product, Bio-Gel HTP (Bio-Rad Labs., Richmond, CA, U.S.A.) was treated first with boiling dilute sodium hydroxide and then with dilute phosphate buffers according to the procedure described by Bernardi[9].

Chromatography was carried out for the most part as previously described[1,8]. The hydroxyapatite columns were 10 × 2.5 cm. This length is adequate and convenient and reduces the time required for chromatography. Two new eluents, A_1 and D, were used giving a total of six eluents (Table I). The preparation to be chromatographed was dialyzed against eluent A_1 for approximately 30 h, and a sample containing approximately 50 mg of protein was applied to the column. Each effluent fraction was given the same designation as the solvent which eluted it. Protein concentration was calculated from transmittance at 279 nm, with an absorptivity of 0.543 $cm^{-1}\ g^{-1}\ l$, the value reported for monomeric myosin[10]. Effluents were consistently clear, and no correction was made for light scattering.

Gel electrophoresis

Sodium dodecyl sulfate (SDS) was of technical grade, purchased from Matheson, Coleman, and Bell (East Rutherford, NJ, U.S.A.). Alkyl chains longer than C_{12} are present and may be important in the dissociating action of this detergent. Enzyme grade acrylamide and N,N′-methylenebisacrylamide, both specified as suitable for acrylamide gel electrophoresis, were products of Eastman-Kodak (Rochester, NY, U.S.A.).

Gel electrophoresis was carried out in tubes (65 × 4.5 mm I.D.) essentially as described by Weber and Osborn[11].

Enzymatic activity assays

Adenylate deaminase activity was determined by the method of Nikiforuk and Colowick[12], creatine phosphokinase by the method of Bernt and Bergmeyer[13], and aldolase by the method of Lowry[14]. Pyruvate kinase activity was measured as described by Adam[15] for the assay of ADP, except that ADP was supplied in excess and the unknown solution served as the only source of pyruvate kinase. ATPase activity was determined by the method of Fritz and Hamrick[16] adapted for continuous assay of column effluent.

RESULTS AND DISCUSSION

Typical chromatographic patterns given by myosin preparations made from fresh and aged bovine muscle are shown in Fig. 1. The major points of interest are the absence of fractions B_2, C and D from fresh muscle and the decrease in B_1, the major myosin-containing fraction, on aging. The results are similar to those obtained with rabbit muscle. None of the fractions from fresh muscle myosin preparations made from either bovine or rabbit muscle has any actin (Fig. 2a); however, with preparations made from muscle aged at 0° or 4°C (Fig. 2b), actin appears in fractions C and D, as well as in the fractions, A_2 and A_2', eluted by eluent A_2 (see below).

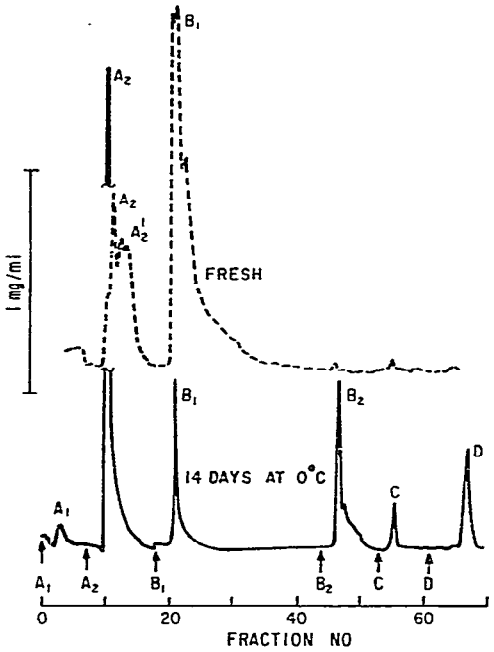

Fig. 1. Chromatograms of myosin preparations made from fresh bovine muscle and from bovine muscle aged at 0°C for 14 days. The arrows indicate the points at which elution with the indicated eluents was initiated. Chromatographic medium: hydroxyapatite. Each fraction contained 12 ml.

The gel electrophoretic patterns show that fraction B_1 always contains myosin, but does not contain actin even when actin is present in the sample applied to the column (Fig. 2b). Small amounts of other components are usually present in B_1. One of these appears immediately below the heavy chain of myosin and, on the basis of its mobility, is tentatively identified as the B′ protein of D'Albis[17], which has a molecular weight slightly less than that of the heavy chain of myosin. Small amounts of unidentified components with molecular weights above 205,000 are also frequently present.

The significance of the fractions eluted by eluent A_2 is nuclear. In previous work[1] the protein applied to the column was dissolved in what is here designated as

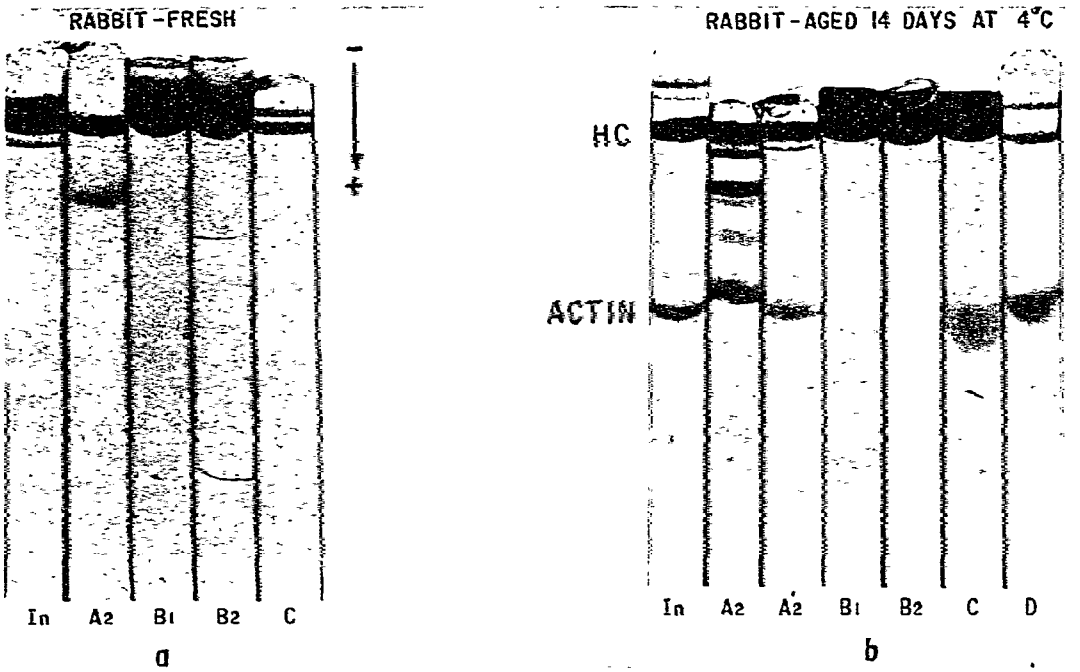

Fig. 2. Gel electrophoretic patterns obtained with proteins dissociated in SDS + urea. The direction of migration of anionic species is indicated by the arrow. (a) SDS gels of a myosin preparation made from fresh rabbit muscle and of fractions obtained from it by chromatography on hydroxyapatite. (b) SDS gels of a myosin preparation made from rabbit muscle aged at 4°C for 14 days and of fractions obtained by chromatography on hydroxyapatite. Note the presence of actin in the sample applied to the column (In) and in fractions A_2, A_2', C, and D. The components of fraction A_2 were retarded, probably because of a faulty gel. Another sample of the same fraction run simultaneously showed no such retardation; the actin lined up with that in the other gels. HC = heavy chain(s) of myosin.

eluent A_2, and this was the first solution used to elute the column. Buffer A_2 is 0.16 *M* in phosphate, whereas the solution now used as sample solvent and first eluent, eluent A_1, is 0.05 *M* in phosphate. Virtually all of the protein in the sample of myosin preparation applied to the column is, in the current procedure, adsorbed onto the hydroxyapatite; little or no material is eluted in fraction A_1 (Fig. 1). The protein which in the present elution program comes out in fraction A_2 was formerly not adsorbed to the column. The composition of fraction A_2 is variable, and it frequently emerges as a doublet ($A_2 + A_2'$). A_2 contains myosin and at least one other component (Fig. 2a), possibly C-protein[18]. With aged muscle, fraction A_2 also contains actin (Fig. 2b) and several components only tentatively identified, including the B-protein of Starr and Offer[19].

The protein in myosin preparations made from aged muscle binds more tightly to the exchanger, hydroxyapatite. As aging proceeds, progressively less protein is eluted in fraction B_1, and progressively more in fractions B_2, C and D; other protein remains so tightly bound that it is not eluted even by sodium hydroxide. In a typical case, after prolonged aging *viz.*, 20 days at 0°C, B_1 decreased to *ca.* 20% of its value

for fresh muscle. When chromatography on hydroxyapatite was first applied to myosin preparations, it was found that when a solution of myosin is stored prior to chromatography a similar increase in tightness of binding of the protein occurs. There was also independent evidence of aggregation in the stored solutions[8]. Since similar changes in chromatographic behavior occur when myosin preparations are stored and when muscle is aged prior to making the myosin preparation, the effects observed on aging were initially attributed to aggregation of the myosin[1]. Although aggregation most likely occurs, the work described here shows that this explanation was an over-simplification.

During aging there is a breakdown in the structure of the Z-disc to which the thin filament is attached, a process at least partially attributable to the calcium activated factor[20,21]. This process releases actin from its attachment to the Z-disc so that, in aged muscle, it is extracted together with myosin. When actin is present in the sample applied to hydroxyapatite, the recovery of protein in fraction B_1, which is free of actin, diminishes, and actin and myosin appear in fractions C and D; some myosin, with or without actin, is also eluted in fraction B_2 (Fig. 2b). The collective recovery in fractions A_1–C is also reduced in the presence of actin. The observations on aging muscle thus reflect the detachment of the thin filament from the Z-disc. In addition to degradation of the Z-disc, some proteolytic digestion of myosin may occur[22], as well as aggregation of the heavy chains of myosin.

As a further measure of the efficiency of the chromatographic procedure described for the purification of myosin, a "soluble protein preparation" of rabbit muscle (see Materials and methods) containing both sarcoplasmic and myofibrillar proteins was subjected to chromatography on hydroxyapatite. The distribution of four selected enzymatic activities in fractions A_1–C, of ATPase in fractions A_2–C and of protein were determined (Table II). A_1 was not assayed for ATPase activity; this fraction is expected to contain ATPases other than myosin. The low total recovery of ATPase, 57%, is at least partially attributable to the fact that this figure does not include the activity in fraction A_1. Fraction D, being strongly alkaline, and of which

TABLE II

DISTRIBUTION OF ACTIVITY AND PROTEIN AMONG THE ELUENT FRACTIONS

	Activity (%) in fraction*					*Total recovery (%)** from column*
	A_1	*A_2*	*B_1*	*B_2*	*C*	
Enzymatic activity						
AMP Deminase	30.0	34.0	0	0	36.0	124.0
Pyruvate kinase	42.0	58.0	0	0	0	135.0
Creatine phosphokinase	37.0	63.0	0	0	0	98.0
Aldolase	30.0	25.0	29.0	9.0	7.0	>100.0
ATPase	—***	9.0	77.0	8.0	6.0	57.0
Protein	28.0	25.0	36.0	7.0	4.0	76.0

* The total recovery of each activity and of protein in fractions A_1–C, was here taken as 100%. For ATPase, the total activity recovered in fractions A_2–C was taken as 100%.

** Activity or protein eluted in fractions A_1–C (for ATPase, A_2–C) relative to that applied to the column. Recoveries over 100% were assumed to result from the presence of inhibitors in the applied sample which were removed from effluents A_1–C.

*** Not determined.

little or none appears with preparations from fresh muscle, was not included in the recovery calculations. In contrast to the elution pattern with myosin preparations, where little or no protein emerges in fraction A_1, in the case of the soluble protein preparation more than one-fourth of the protein recovered from the column was not adsorbed to it and was eluted in this fraction (Table II). Fractions A_1 and A_2 contained substantial amounts of each of the four enzymatic activities for which both of them were assayed.

As also shown in Table II, AMP deaminase is absent from the major fraction B_1, as well as from B_2. (B_1 is the fraction of major interest because it consists predominantly of myosin and is free of actin, even when the preparation applied to the column contains a large amount of the latter.) Deaminase is known to bind tightly to myosin and to be dissociated from it by phosphate[23]. Chromatography on DEAE-Sephadex A-50 by the well-known procedure of Richards *et al.*[4] does not remove all deaminase from myosin. The efficiency of hydroxyapatite in removing this protein may be related to the use of phosphate at high ionic strength for the chromatography.

Pyruvate kinase and creatine phosphokinase were also removed completely from fractions B_1 and B_2. Isozymes of creatine phosphokinase are located in the cytosol, the mitochondria, and the myofibrils[24]. The myofibrillar isozyme binds tightly to myosin[25].

Aldolase was not removed from fraction B_1 (nor from B_2). As is evident from the ratio of aldolase activity to protein content for each fraction (Table II), the specific activity of aldolase does not vary greatly among the five fractions A_1–C.

The above results demonstrate (1) the efficiency of the fractionation procedure for the removal from myosin preparations of myofibrillar and sarcoplasmic protein contaminants, including actin, creatine phosphokinase, pyruvate kinase, and AMP deaminase, but not FDP aldolase; and (2) that on aging muscle, simultaneous with the presumed aggregation of myosin, there are changes in the composition of some of the fractions, in particular the appearance of actin. However, the major myosin-rich fraction remains free of actin.

In addition to the purification provided by chromatography of myosin on hydroxyapatite, the procedure has the following advantages: (1) The support medium has a rather high density and is dimensionally very stable. (2) The length/diameter ratio is small, and the flow-rate is good. (3) The composition of the sample buffer and the eluents favors the solubility and stability of myosin. Thus, the pH is neutral (6.8), the minimum ionic strength is 0.5, and chloride and phosphate are both present, the former at a concentration of 0.4 *M*, the latter at a minimum concentration of 0.05 *M*.

In our hands the procedure for chromatography on DEAE-cellulose[3] was found to be troublesome. The low ionic strength (0.21) and the lack of ions such as phosphate lead to low solubility and, sometimes, slow precipitation of myosin. The use of pyrophosphate, as in the method utilizing DEAE-Sephadex[4], avoids these problems. However, for enzymatic studies the authors recommend against the use of pyrophosphate[4]. Furthermore, we have found it difficult to remove pyrophosphate from myosin completely. For enzymatic studies 0.15 *M* phosphate was recommended as a replacement for pyrophosphate, but the authors had only limited experience with this medium[4]. In any case, DEAE-Sephadex is not as stable dimensionally as hydroxyapatite, and neither of the DEAE exchangers removes deaminase completely.

Methods utilizing affinity chromatography are, in general, much superior to

other chromatographic procedures with regard to selectivity. However, interaction of a chromatographic support medium with a given protein does not ensure removal of a contaminating protein; a complex of the desired protein with its contaminant may bind to the support medium. Therefore, the ability of the procedure to remove the contaminant must be demonstrated by analysis, particularly in the case of a protein such as myosin which interacts strongly with several other proteins. Unfortunately, it appears that no attempt was made to prove the absence of contaminants such as pyruvate kinase and deaminase from myosin preparations after affinity chromatography on immobilized actin and immobilized nucleotides[5–7]. Therefore, the relative efficacy of affinity chromatography and chromatography on hydroxyapatite in this regard cannot be evaluated.

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