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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF MYOSIN'S SUBFRAGMENT 1 AND LIGHT CHAINS ON SPHEROGEL TSK-TYPE COLUMNS

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SUMMARY

Myosin's subfragment 1 and three light chains were chromatographed rapidly at room temperature on Spherogel TSK-type columns, recently developed for steric-exclusion high-performance liquid chromatography, with no adsorption of the proteins to the columns or loss of enzymic activity. Crude preparations of subfragment 1 were extensively purified on Spherogel TSK SW-3000 columns. Unresolved α-chymotryptic-subfragment 1 was separated into its two isoenzymes despite only a small difference in molecular weights, presumably because of conformational differences. Light chain 2 was purified from a mixture of all three light chains by chromatography on a Spherogel TSK SW-2000 column; light chains 1 and 3 eluted as two distinct complexes having ratios of 2:1 and 1:1.

INTRODUCTION

Contractile proteins such as myosin, its enzymatically active subfragments [subfragment 1 (S1)** and heavy meromyosin (HMM)], or its three light chains (lc_1, lc_2, and lc_3) are typically purified from crude protein preparations by steric-exclusion chromatography[1], ion-exchange chromatography[2,3], or, most recently, affinity chromatography[4,5], all on columns filled with carbohydrate gels. Unfortunately, these gels are extremely fragile to changes in pressure, pH, and ionic strength[6]. The mandatory slow flow-rates and gentle pH or ionic gradients result in slow separations, typically 15–20 h for myosin, its subfragments, or its light chains[1–3].

Conventional steric-exclusion chromatography is not only time consuming, but also completely inadequate for resolving the two isoenzymes of α-chymotryptic S1

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** Abbreviations used: S1 = subfragment 1; act-S1 = α-chymotryptic subfragment 1; lc_1 = light chain 1; lc_2 = light chain 2; lc_3 = light chain 3; act-S1 (lc_1) = α-chymotryptic subfragment 1 isoenzyme containing light chain 1; act-S1 (lc_3) = α-chymotryptic subfragment 1 isoenzyme containing light chain 3; HMM = heavy meromyosin; act = α-chymotrypsin; DTT = DL-dithiothreitol (Cleland's Reagent).

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(αct-S1), αct-S1 ($1c_1$) and αct-S1($1c_3$), or separating myosin's three light chains; these proteins are too similar in size. Affinity chromatography supports capable of such specificity have yet to be developed. Fortunately, conventional ion-exchange chromatography may be used to resolve αct-S1's isoenzymes or the light chains on the basis of charge differences but, again, 15–20 h are required[2,3].

Until recently, most efforts at speeding up protein separations by using fast flow-rates, *i.e.*, high column pressures, and pressure-stable silica supports in high-performance liquid chromatography (HPLC) systems have been only marginally successful. Steric-exclusion and ion-exchange HPLC supports have been tested, but the reactive silica surface of these supports irreversibly adsorbs and/or denatures many proteins[7]. Recently, efficient separations of serum proteins by steric-exclusion HPLC have been reported using a support which consists of a chemically-modified silica, with little adsorption and no apparent denaturation occurring[8,9].

The next step is to test the usefulness of steric-exclusion HPLC for biologically important proteins, most of which remain to be tested. In this study, we tested columns filled with the recently marketed Spherogel TSK-type steric-exclusion HPLC-support, a pressure-stable, hydrophilic, chemically-modified silica, for purifying myosin's subfragments (S1 and HMM), and myosin's three light chains ($1c_1$, $1c_2$, and $1c_3$), in an attempt to speed up existing purification methods. We evaluated the Spherogel TSK-type columns on the basis of their separating powers and the recovery of protein and enzymic activity, specifically S1's ATPase activities and $1c_2$'s ability to be phosphorylated before and after HPLC. In the process of analyzing the columns' separating powers, we also examined the chromatographic properties of S1 and myosin's light chains on the Spherogel TSK-type columns in the hope of elucidating some of the proteins' hydrodynamic properties, *e.g.*, their conformations in solution and possible interactions with each other.

EXPERIMENTAL

Protein preparation

All of the proteins used in the HPLC analyses were extracted from the white skeletal muscle of rabbit. Myosin was purified by the method of Tonomura *et al.*[10] as modified by Crooks and Cooke[11]. Heavy meromyosin was purified by the method of Weeds and Taylor[2]. Myosin subfragment 1 (S1) was purified either by papain digestion of myofibrils by the method of Cooke[1] or by α-chymotryptic digestion of myosin by the method of Weeds and Taylor[2]; the crude, unresolved S1 applied to the HPLC column consisted of the S1-containing supernatant solution after sedimentation of papain-digested myofibrils or α-chymotrypsin-digested myosin. The two isoenzymes of αct-S1, αct-S1($1c_1$) and αct-S1($1c_3$), used as HPLC standards, were separated by ion-exchange chromatography[2]. A fraction containing all three of myosin's light chains was isolated from myosin as described by Holt and Lowey[3]. An aliquot of this light chain mixture was fractionated into pure $1c_2$ and a mixture of $1c_1$ and $1c_3$ by Blue Sepharose affinity chromatography according to the method of Toste and Cooke[4]. All of the proteins were dialyzed against the HPLC column buffer, 50 m*M* sodium phosphate (pH 7.4), 0.2 *M* ammonium sulfate, 1 m*M* ethylene diaminetetraacetic acid (EDTA), 0.2 m*M* dithiothreitol (DTT), and 0.02% sodium azide for 3 h before HPLC.

HPLC analysis

Two Spherogel TSK-type columns (Altex Scientific) were used for the HPLC analyses. S1 and HMM were chromatographed on a Spherogel TSK SW-3000 column (600 × 7.5 mm). Myosin's light chains were chromatographed on a Spherogel TSK SW-2000 column (600 × 7.5 mm). Each protein preparation was recycled through the column three more times in an attempt to improve the separation of individual proteins.

Each Spherogel TSK-type column was calibrated by chromatographing protein standards and plotting the log mol.wt. of each standard against its retention time. Ferritin (mol.wt. 450,000), aldolase (mol.wt. 158,000), hexokinase (mol.wt. 104,000), bovine serum albumin (BSA) (mol.wt. 68,000), ovalbumin (mol.wt. 45,000), chymotrypsinogen A (mol.wt. 25,000), and cytochrome *c* (mol.wt. 12,500) were chromatographed on the SW-3000 column; aldolase, hexokinase, BSA, ovalbumin, chymotrypsinogen A, and cytochrome *c* were chromatographed on the SW-2000 column. The columns were equilibrated with the HPLC column buffer at room temperature before the proteins were chromatographed. The contractile proteins and the protein standards were then chromatographed at room temperature in the HPLC column buffer at flow-rates ranging from 12–60 ml/h.

Each Spherogel TSK-type column was attached to an Altex Model 320 advanced research chromatograph consisting of a Model 100A dual piston analytical pump (flow-rate accuracy of ±1%, or 0.005 ml/min, up to 10,000 p.s.i.), a Model 153 UV detector, a Model 210 injection valve and a Model 155 recorder. The injection valve was replaced by a Model 100A recycle chromatography port for the recycle chromatography. The low piston displacement volume (0.1 ml) of the 100A pump, makes it especially well-suited for recycle chromatography.

Assays

The protein recoveries and purity of the HPLC-eluted proteins were determined by assaying their concentrations and composition before and after HPLC. The protein concentrations of the protein preparations and the HPLC-eluted proteins were measured spectrophotometrically at 595 nm with the Bradford assay[12], a protein specific assay which is based on the binding of the dye Coomassie brilliant blue G-250 to proteins. Simple absorption measurement of protein concentration at 230 or 280 nm was judged too non-specific for our purposes; UV absorbance by non-proteins, *e.g.*, nucleotides, masked the extent of the protein separations achieved by HPLC. The protein composition of the protein preparations and the HPLC-eluted proteins was determined by gel electrophoresis according to the method of Ames[13]. We used 1.5-mm slabs of polyacrylamide–BIS–SDS and the discontinuous buffer system of Laemmli[14]. The protein bands on the Coomassie blue-stained gels corresponding to myosin's light chains and S1 light and heavy chains were quantitated by densitometry; the dried gels were scanned at 550 nm.

The recovery of the proteins' enzymic activity after HPLC was measured by assaying S1's ATPase activities and $1c_2$'s phosphorylation activity. S1's ATPase activities were measured colorimetrically by the method of Martin and Doty[15]. The "K^+-ATPase" activity was assayed in 0.6 *M* potassium chloride, 1 m*M* EDTA, and 1 m*M* adenosine triphosphate (ATP) at pH 8, 25°C; "Ca^{2+}-ATPase" activity in 0.6 *M* potassium chloride, 4 m*M* calcium chloride, and 1 m*M* ATP at pH 8, 25°C; "Mg^{2+}-

ATPase" activity in 50 m*M* potassium chloride, 1 m*M* magnesium chloride, and 1 m*M* ATP at pH 8, 25°C; and actin-activated ATPase activity in 30 m*M* potassium chloride, 2 μM actin, 4 m*M* magnesium chloride and 1 m*M* ATP at pH 8, 25°C. lc_2 was phosphorylated as described by Pires and Perry[16] using myosin's light-chain kinase purified in Dr. J. T. Stull's laboratory (Department of Pharmacology, University of Texas, Dallas, TX, U.S.A.) from skeletal muscle. lc_2's phosphorylation activity was determined by gel electrophoresis on 7.5% polyacrylamide–urea slab gels by the method of Pires and Perry[16]. The protein bands on the Coomassie blue-stained gels corresponding to phosphorylated and unphosphorylated lc_2 were quantitated by densitometry.

Materials

The α-chymotrypsin and papain used in the S1 preparations were purchased from Sigma (St. Louis, MO, U.S.A.) and Worthington (Freehold, NJ, U.S.A., respectively. The protein standards used to calibrate the Spherogel TSK-type columns were obtained as a kit from Boehringer (Mannheim, G.F.R.). All other reagents were of analytical reagent-grade or of the best grade available.

RESULTS

Chromatographic properties

Protein standards. All of the protein standards tested were retarded by the Spherogel TSK SW-3000 column (flow-rate, 60 ml/h) to some extent, eluting at the following times: ferritin, 9.5 min; aldolase, 12.6 min; hexokinase, 13.8 min; BSA, 15.2 min; ovalbumin, 16.3 min; chymotrypsinogen, 18.2 min and cytochrome *c*, 20.3 min. Plotting the log mol.wt. of each protein against its retention time (min) yielded an absolutely straight line, giving the TSK SW-3000 column a mol.wt. separation range of at least 12,500–450,000. The protein standards eluted from the Spherogel TSK SW-2000 column (flow-rate, 60 ml/h) at the following times: ferritin, 10.9 min; aldolase, 10.9 min; hexokinase, 10.9 min; BSA, 11.8 min; ovalbumin 13min; chymotrypsinogen, 14.6 min and cytochrome *c*, 16.5 min. Ferritin, aldolase, and hexokinase were occluded from the TSK SW-2000 column; they all eluted in the column's void volume. The rest of the protein standards were retarded to some extent; plotting the log mol.wt. of these standards *vs.* their retention time (min) yielded an absolutely straight line, giving the TSK SW-2000 column a mol.wt. separation range of approx. 12,500–80,000.

S1 and HMM. Papain-S1 and αct-S1 each eluted from the Spherogel TSK SW-3000 column as a single peak at column flow-rates of 30 ml/h or faster. Papain-S1 eluted slightly earlier than unresolved αct-S1 (11.78 min *vs.* 13.03 min, at a flow-rate of 60 ml/h). Comparison of these retention times with the calibration curve for the SW-3000 column yielded effective mol.wts. of 204,000 and 137,000 for papain-S1 and αct-S1, respectively. When the flow-rate was slowed to 12 ml/h, papain-S1 still eluted as a single peak, but αct-S1 separated into its two isoenzymes (Fig. 1). Peak a eluted at 63 min, ocrresponding to an effective mol.wt. of 158,000; peak b eluted at 64.8 min, corresponding to an effective mol.wt. of 140,000. Gel electrophoresis indicated that peak a consisted of S1 (lc_1) and peak b consisted of S1 (lc_3). In contrast, the protein composition across the papain-S1 peak (chromatographed at

12 ml/h) was homogeneous, with respect to both S1 heavy chains and to its light chains Recylcing both S1's through the SW-3000 column three more times did not improve the separations.

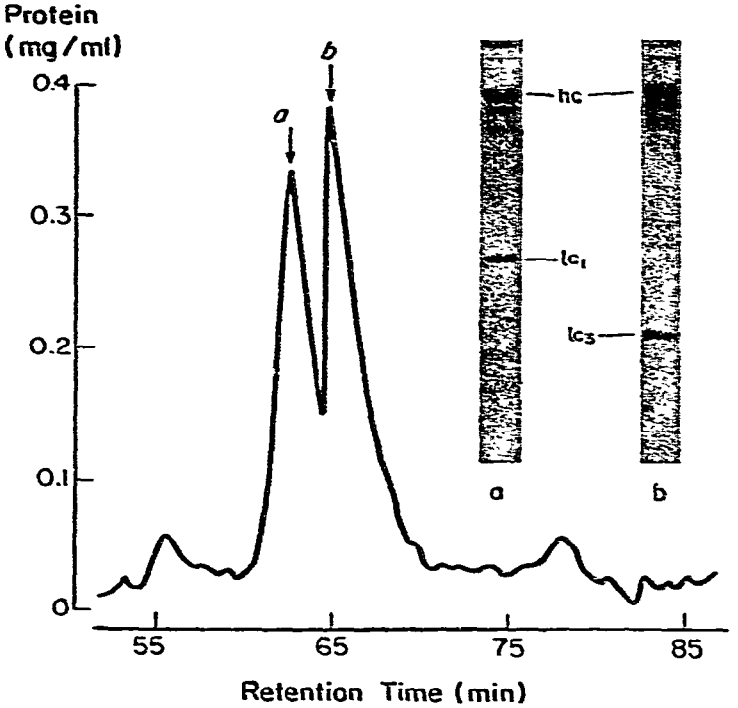

Fig. 1. HPLC separation of α-S1 isoenzymes, α-S1 (1 mg, 2.5 mg/ml) was applied at room temperature to a Spherogel TSK SW-3000 column (600 × 7.5 mm) that had been equilibrated with the HPLC column buffer, 50 m*M* Na_3PO_4 (pH 7.4), 0.2 *M* $(NH_4)_2SO_4$, 1 m*M* EDTA, 0.2 m*M* DTT and 0.02% NaN_3. Fractions (300 μl each) were collected at a flow rate of 12 ml/h. The protein concentration of each fraction was monitored at A_{595nm} by the Bradford assay[12]. Figure inset: Polyacrylamide–SDS gel profile of the HPLC-resolved α-S1 peaks. The positions of α-S1's heavy chain (hc) and two light chains (lc_1 and lc_3) are noted. Aliquots of the top fraction (a and b) from each protein peak were prepared for gel electrophoresis[14], from which 40 μl (10 μg protein) were applied to a 12.5% polyacrylamide–0.1% SDS gel[13].

HMM eluted in the column's void volume, indicating that it was completely occluded from the Spherogel TSK SW-3000 column.

Light chains. HPLC of the preparation containing all three of myosin's light chains on the Spherogel TSK SW-2000 column at higher flow-rates, *e.g.*, 60 ml/h, yielded a single, poorly-resolved peak containing 2 distinct shoulders. HPLC of the same preparation at 12 ml/h yielded 3 peaks (Fig. 2). Peak a eluted at 61.6 min, peak b at 68.5 min, and peak c at 73.9 min, corresponding to effective mol.wts. of 58,000, 36.000, and 23,500, respectively. Gel electrophoresis of aliquots from the column fractions across the 3 peaks revealed that peak a consisted mainly of lc_1, with some lc_3 present, and peak b consisted mainly of lc_3, with a considerable amount of lc_1 also present, but peak c consisted almost exclusively of lc_2. Qualitative examination of the gel suggested that peaks 1 and 2 consisted of two distinct lc_1–lc_3 complexes.

Densitometry of the gel confirmed this suggestion: the protein composition across peak a consisted of a 2:1 complex of lc_1 and lc_3 and peak 2 consisted of a 1:1 complex. Recylcing the light-chain peaks through the SW-2000 column three more times did not improve the separations.

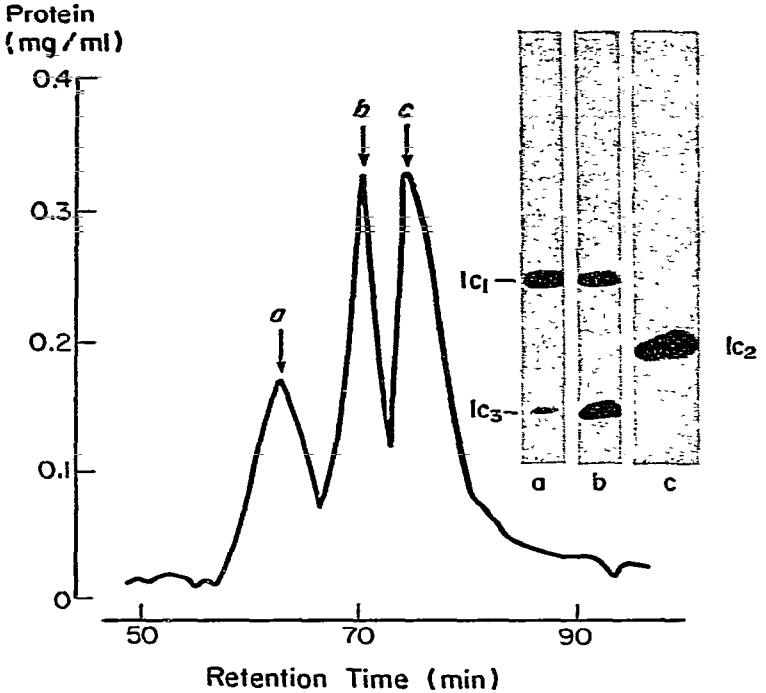

Fig. 2. HPLC separation of a mixture containing myosin's three light chains. The light-chain mixture (2 mg, 4 mg/ml) was chromatographed on a Spherogel TSK SW-2000 column as described in Fig. 1. Figure inset: Polyacrylamide–SDS gel profile of the HPLC-resolved light-chain peaks. Aliquots of the top fraction (a, b and c) from each protein peak were prepared for gel electrophoresis[14], from which 40 μl (6–10 μg protein) were applied to a 15% polyacrylamide–0.1% SDS gel[13].

HPLC of a mixture of lc_1 and lc_3 and HPLC of a sample of lc_2, both separated from the original light-chain preparation by Blue Sepharose chromatography, gave the same results as the HPLC of the mixture of all three light chains. The mixture of lc_1 and lc_3 chromatographed as two peaks, corresponding to peaks a and b of the mixture of all three light chains. lc_2 eluted exactly like peak c of the mixture of all three light chains.

Purification

HPLC of crude papain-S1 and act-S1 yielded highly purified S1's. Comparison of gel electrophoresis profiles of papain-S1 before and after HPLC indicated that the HPLC-purified papain-S1 lacked numerous protein contaminants present in the crude papain-S1 preparation, specifically actin and other proteins; these other proteins appeared to be proteolytic fragments of myosin's heavy chains (Fig. 3A). HPLC-

purified act-S1 (Fig. 3B) lacked several heavy-chain fragments present in crude act-S1. The protein recoveries from the Spherogel TSK-type columns were 96–98% for all of the proteins studied.

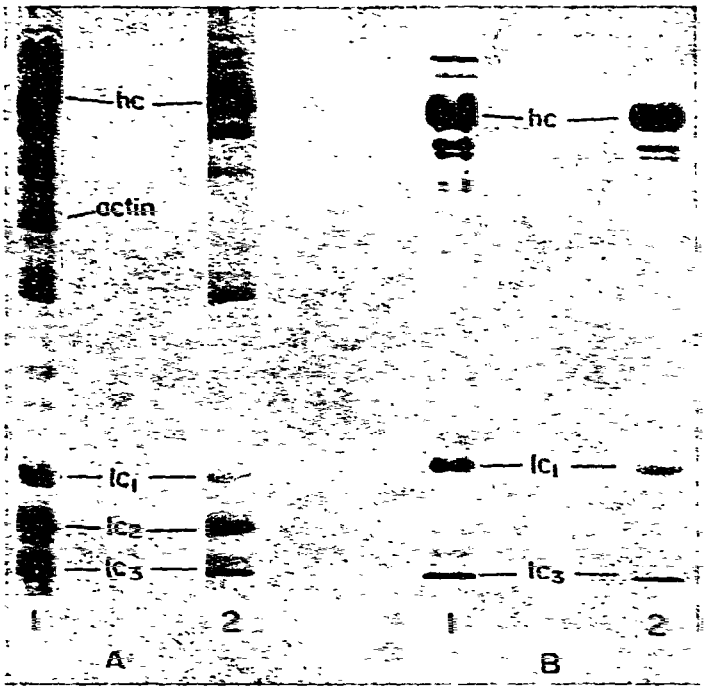

Fig. 3. Polyacrylamide–SDS gel profiles of S1 purified by HPLC. Crude papain-S1 and act-S1 (2 mg each) were chromatographed separately on the Spherogel TSK SW-3000 column at 60 ml/h in the HPLC column buffer. Protein recoveries from each column run were 96–98%. Each purified S1 was collected as a single fraction. Aliquots of each S1 (10–15 μg) were set aside before and after HPLC and prepared for gel electrophoresis (see Fig. 1). (A) Papain-S1: (1) before HPLC, crude papain-S1 consisted of papain-S1's heavy chains (hc) and light chains (lc) (85.2%), actin (7.2%), and proteolytic fragments (7.6%); (2) after HPLC, purified papain-S1 consisted almost exclusively of papain-S1 heavy chains (hc) and its three light chains. (B) act-S1: (1) before HPLC, crude act-S1 consisted of act-S1's heavy chains and light chains (89%) and proteolytic fragments (11%); (2) after HPLC, purified act-S1 consisted almost entirely of act-S1 heavy chains (hc) and its two light chains.

The ATPase activities (in μmol/g·sec) of the HPLC-purified S1's were "K^+-ATPase", 80–97; "Mg^{2+}-ATPase", 0.1; "Ca^{2+}-ATPase", 9; and actin-activated ATPase (1 μM actin), 5.8–6.4.

Only peak c of the HPLC chromatogram of the mixture of all three light chains (Fig. 2) consisted of a purified, single component, lc_2. SDS- and urea-gel electrophoresis indicated that the HPLC-lc_2 was intact and was completely phosphorylated by myosin's light-chain kinase.

The Spherogel TSK-type columns used to purify S1 and the light chains (600 × 7.5 mm) were not designed for HPLC on a preparative scale *per se*. Nevertheless, 40 mg of an equimolar mixture of the protein standards used to calibrate the

Spherogel TSK SW-3000 and SW-2000 columns were chromatographed on the two columns without any apparent overloading or loss of resolution.

DISCUSSION

Steric-exclusion HPLC promises to be an excellent way of purifying contractile proteins rapidly, for two reasons. First, the separating powers of the Spherogel TSK-type columns were quite impressive. Extraneous proteins were separated from both papain-S1 and *act*-S1 on the Spherogel TSK SW-3000 column within 13 min, yielding highly purified S1's (Fig. 3). Even the purified protein preparations were further separated by HPLC. The two isoenzymes *act*-S1(lc_1) and *act*-S1(lc_3) were separated from unresolved *act*-S1 (Fig. 1) and lc_2 was separated from a mixture of all three light chains (Fig. 2), despite mol.wt. differences of only 8% between the two S1 isoenzymes and of 11% between lc_2 and lc_1 and 14% between lc_2 and lc_3 (see refs. 3 and 17 for mol. wts.). The HPLC separations of both *act*-S1 and the light chains compare favorably with separations achieved by ion-exchange chromatography[1–3,17], but require only a fraction of the time (70–80 min *vs.* 15–20 h). Conventional steric-exclusion chromatography using sepharose or sephadex is completely inadequate for separating proteins of comparable size like *act*-S1's isoenzymes and myosin's light chains.

A second reason for using steric-exclusion HPLC to purify contractile proteins is that the recovery of protein and enzymic activity from the Spherogel TSK-type columns was excellent. The protein recoveries of all the HPLC-purified proteins were 96–98%, indicating that S1 and myosin's light chains did not adsorb to the Spherogel TSK-type columns. The recovery of ATPase activities was quite good, indicating that S1 was not denatured. Comparison of the ATPase activities of the HPLC-purified S1 (see Results) with those of S1 purified in our lab by conventional ion-exchange chromatography indicates that the HPLC-purified S1's "K^+-ATPase" activity was 28% higher, the "Mg^{2+}-ATPase" and actin-activated ATPase activities were identical, and the "Ca^{2+}-ATPase" activity was 18% lower. The ATPase activities of the HPLC-purified S1 also compare well with published values[2]. The light chains also appeared to be undamaged by the HPLC. The light chains' electrophoretic mobilities remained unchanged after HPLC, and the HPLC-purified lc_2 was completely phosphorylated by myosin's light-chain kinase.

The reason that no enzymic activity was lost even though S1 and the light chains were chromatographed at room temperature is undoubtedly that the proteins remained in the Spherogel TSK-type columns for short times. In contrast, the long residence times of proteins on conventional ion-exchange and steric-exclusion columns make it necessary to work at cold-room temperatures, *e.g.*, 4°C.

In addition to being useful for purifying contractile proteins, steric-exclusion HPLC is useful for characterizing the proteins' conformations in solution. The HPLC-derived mol.wts. on the unresolved *act*-S1 (137,000) and particularly the papain-S1 (204,000) are significantly higher than S1's accepted mol.wt. of 115,000–120,000[1,2,17], suggesting that S1 is elongated, which makes it behave like a larger, spherical protein on the Spherogel TSK SW-3000 column. This explanation agrees well with direct evidence that S1 is an elongated ellipsoid[17].

S1's HPLC-derived mol.wts. suggest conformational differences between

papain-S1 (204,000) and unresolved act-S1 (137,000) and between act-S1(lc_1) (158,000) and act-S1(lc_3) (140,000). The differences in mol.wt. between papain-S1 and unresolved act-S1 (49%) and between act-S1(lc_1) and act-S1(lc_3) (13%) are significantly greater than those predicted from the known mol.wts. of myosin's light chains (16 and 5%, respectively). A number of explanations are possible for these unexpected high differences. Perhaps the differences in chromatographic behavior are due to differences in the S1's light-chain complements which result in conformational differences among the various S1's. Or perhaps, the exaggerated differences in mol.wts. between papain-S1 and unresolved act-S1 are due to conformational differences between the two S1's induced by differences in papain's and α-chymotrypsin's cleavage of myosin. Finally, the unexpectedly high difference in mol.wts. between act-S1(lc_1) and act-S1(lc_3) could be due to differences in the spatial arrangement of the light chains on the two heads.

The HPLC-derived mol.wts. of myosin's light chains are closer to the reported mol.wts. than are those of the S1's. lc_2's mol.wt. (23,500) is a bit higher than the reported value of 18,000, presumably because lc_2 is elongated[18–20]. Computation of the mol.wts. for lc_1 and lc_3 from the mol.wt. 58,000 2:1 lc_1–lc_3 complex (Fig. 2, peak a), and the mol.wt. 37,000 1:1 lc_1–lc_3 complex (Fig. 2, peak b) yields mol.wts. of 21,000 and 16,000 for lc_1 and lc_3, respectively. These values agree exactly with published values, suggesting, perhaps, that lc_1 and lc_3 adopt nearly spherical shapes when compexed to each other.

Too much emphasis should not be placed on the absolute values of the HPLC-derived mol.wts. of the various S1's and myosin's light chains because of the acknowledged non-spherical shapes of these proteins. The exaggerated differences in mol.wts. between papain-S1 and act-S1 and particularly between act-S1(lc_1) and act-S1(lc_3), however, are noteworthy and strongly suggest conformational differences between the different S1's.

We have shown that steric-exclusion HPLC can be very useful for purifying and characterizing S1 and myosin's light chains. Other types of HPLC appear promising as well, but remain to be tested. Further refinement of ion-exchange HPLC, specifically the elimination of protein adsorption and denaturation from the ion-exchange supports, should prove invaluable to separations of S1 and of light chains, in that conventional ion-exchange chromatography is superior to conventional steric-exclusion chromatography for separating these proteins[2,3]. Unlike ion-exchange HPLC, reversed-phase HPLC is not likely to be useful for S1 chromatography because the acidic pH values and organic solvents used with reversed-phase HPLC generally denature proteins; but reversed-phase HPLC may prove an excellent way to separate myosin's light chains. They appear inherently more stable than S1 because they can be extracted from myosin by urea or guanidine–HCl denaturation, followed by alcohol precipitation[3]. It seems unlikely, therefore, that elution of the light chains on a reversed-phase column with a 10% methanol–water eluent, for example, would denature them further. Smaller proteins, comparable in size to the light chains, have proven amenable to reversed-phase HPLC analysis[21], whereas larger proteins like S1 tend to adsorb to reversed-phase HPLC supports[22]. It is difficult, of course, to predict exactly how successful these specific approaches will prove. Nevertheless, the potential of HPLC as a rapid, efficient method of separating proteins, particularly contractile proteins, is significant.

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