Journal of Chromatography, 572 (1991) 59–71
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6083

Comparison of ion-exchange chromatography, isoelectric precipitation and reversed-phase high-performance liquid chromatography for the separation of individual cardiac myosin light chains

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(First received April 17th, 1991; revised manuscript received July 22nd, 1991)

ABSTRACT

Three modified procedures for the separation of cardiac myosin light chains are carefully compared. Ion-exchange chromatography gives a purified cardiac myosin light chain 1, whereas light chain 2 is always contaminated by light chain 1. Reversed-phase high-performance liquid chromatography gives the best resolution of these light chains and needs only 20 min for each run. However, it requires pure preparation of myosin light chains before separation. Isoelectric precipitation is the simplest procedure and suitable for large quantities of material. Although it gives the highest yield the separation is not adequate. A modified and rapid procedure for the isolation of cardiac and skeletal total myosin light chains is also presented.

INTRODUCTION

Myosin is an essential contractile and structural protein of the thick filaments of muscle cells. It is well established that myosin consists of two heavy chains (HC) and two pairs of light chains (LC) [1]. Numerous isoforms of myosin exist, not only in different animal species but also in different tissues [2,3]. In the human heart the atrial and ventricular chambers contain distinct myosin molecules that differ in HC and LC structure and ATPase activities [4–6]. The distributions of LC and HC subunits of cardiac myosin have been shown to vary during development and in response to pressure and volume overload [7,8]. It is found that the cardiac myosin light chains can be used as sensitive biochemical markers of irre-

0378-4347/91/$03.50

versible myocardial cell damage [9] and specific probes to characterize the cell composition of different parts of the normal or abnormal heart and to detect the transitions between atrial and ventricular isotypes during development.

There has been a strong interest in finding a way to separate cardiac myosin light chains. The separation of myosin light chains has been achieved by ion-exchange chromatography [10,11], chromatofocusing [12], isoelectric precipitation [13,14], gel permeation [15], reversed-phase high-performance liquid chromatography (HPLC) [16,17], affinity chromatography [18] and preparative polyacrylamide gel electrophoresis (PAGE) [19]. None of these procedures is adequate since all have disadvantages such as poor resolution, time-consuming, low yield or the need for sophisticated equipment.

In this paper we compared and simplified three commonly used procedures for the separation of individual human ventricular myosin light chains. It is found that ion-exchange chromatography is a simple procedure to obtain highly purified LC1, but not LC2. Although reversed-phase HPLC needs sophisticated equipment and pure preparation of myosin light chains before separation it gives the best resolution and is the only way among these three procedures to obtain highly purified LC2. The simplest procedure is isoelectric precipitation which gives the highest yield with relatively poor resolution. We also report here a modified and effective procedure for the isolation and purification of cardiac and skeletal myosin light chains.

EXPERIMENTAL

Preparation of myosin light chains

Human heart and gastrocnemius muscle were obtained 6–12 h after death at autopsy and stored at −70°C. Myosin was prepared from adult human ventricle by a modification of the procedure of Wikman-Coffelt *et al.* [11]. Briefly, the heart tissue was minced and homogenized with 40 m*M* NaCl, 10 m*M* morpholinopropanesulphonic acid (MOPS) pH 7.5, 1 m*M* $MgSO_4$, 2 m*M* ethyleneglycol-bis-N,N'-tetraacetic acid (EGTA), 2 m*M* β-mercaptoethanol (β-MSH) and 1 m*M* phenylmethylsulfonyl fluoride (PMSF). After three washes and centrifugations at 12 000 *g* for 10 min the pellet was dissolved in 0.1 *M* MOPS pH 7.5 containing 2 m*M* EGTA, 2 m*M* β-MSH and 1 m*M* PMSF. NaCl and adenosine triphosphate (ATP) were then added to a final concentration of 600 and 5 m*M*, respectively. After 30 min extraction the supernatant was collected by centrifugation at 27 200 *g* for 15 min. Saturated $(NH_4)_2SO_4$ solution was added to the supernatant and the fraction that was precipitated between 40 and 55% saturation was collected by centrifugation at 12 000 *g* for 10 min. The pellet containing crude myosin was dialyzed overnight against 20 m*M* NaCl, 10 m*M* Tris–HCl pH 7.5, 2 m*M* ethylenediaminetetraacetic acid (EDTA) and 2 m*M* β-MSH. Light chains were prepared from myosin by denaturation in 8 *M* urea and 50 m*M* Tris-HCl pH 7.5 at room temperature for 2 h followed by the addition of 10 volumes of cold 50 m*M*

Tris–HCl pH 7.5 buffer and centrifugation to remove the heavy chain. Solid $(NH_4)_2SO_4$ was added to the supernatant to an 85% saturation to precipiate light chains. The pellet containing crude light chains was dissolved in 1 m*M* EDTA, 10 m*M* Tris–HCl pH 7.5 and 2 m*M* β-MSH and dialyzed against the same buffer overnight. Crude light chains were further purified by isoelectric precipitation. $MgCl_2$ was added to the crude light chains to a final concentration of 10 m*M*. The pH was adjusted to 5.3 with 0.1 *M* citric acid followed by centrifugation at 12 000 *g* for 10 min. The supernatant containing pure light chains was dialyzed against 1 m*M* EDTA, 10 m*M* Tris–HCl pH 7.5 and 2 m*M* β-MSH to remove magnesium ions. All steps were performed at 4°C except where indicated.

Ion-exchange chromatography of myosin light chains

DEAE-Sephadex A-25 was purchased from Pharmacia (Uppsala, Sweden). The column was prepared as described previously [10]. Aliquots (10 ml) of light chains (40 mg) were applied to a 40 cm × 1 cm column which has been washed and equilibrated with 0.05 *M* Tris–HCl pH 7.5. The light chains were eluted from the column by a 160-ml linear gradient of NaCl to 0.7 *M* in 0.05 *M* Tris–HCl pH 7.5 and 1 m*M* EDTA at room temperature. Fractions (2 ml) were collected at a flow-rate of 20 ml/h. Proteins were determined by the Bio-Rad protein assay method using rabbit gammaglobulin as standard or by reading in a Shimadzu Model 160 spectrophotometer at 280 nm. 10% Sodium dodecyl sulfate (SDS) PAGE [20] was employed to analyze selected fractions. The gels were stained with Coomassie Blue [21].

Purification of individual light chains by reversed-phase HPLC

Chemicals. Acetonitrile and 1% trifluoroacetic acid (TFA) in Millipore water were used as buffer solutions. HPLC-grade acetonitrile was purchased from Water Assoc. and sequanal-grade TFA from Pierce (Rockford, IL, USA). Water was glass-distilled and passed through a Milli-Q water system. Both acetonitrile and 1% TFA were filtered and degassed using a Millipore all-glass filter apparatus prior to chromatography.

Apparatus. A Waters Assoc. (Millipore, Milford, MA, USA) HPLC system was used consisting of two Waters Model 510 pumps which were controlled by a Waters interface module and a Digital Professional 350 computer. A Waters U6K injector with a 1-ml injection loop was used for sample injection. A 250-μl Hamilton syringe was employed for loading the loop. Detection was accomplished by using a Waters M490 programmable multi-wavelength detector. Fractions were collected with the help of an LKB-2112 Redirac fraction collector. Reversed-phase separation of ventricular myosin light chains was carried out on a μBondapak C_{18} (150 mm × 3.9 mm I.D.) column (Waters).

Method. Acetonitrile was used as solvent A and 1% TFA in water as solvent B. The column was equilibrated with solvent B. The purified myosin light chain preparation (1 mg) was applied in 0.5 ml of 0.05 *M* Tris–HCl pH 7.5 and separat-

ed at room temperature using a step gradient consisting of (1) 30% A and 70% B for 3 min, (2) 45% A and 55% B for 7 min and (3) 60% A and 40% B for 5 min, at a flow-rate of 1 ml/min. The column was equilibrated with solvent B for 10 min before each run. The detection wavelength was set at 230 nm. Peaks were collected and analyzed by both HPLC and electrophoresis.

Separation of myosin light chains by isoelectric precipitation

Purified light chains in the absence of magnesium ion were subjected to isoelectric precipitation by titration with 0.1 *M* citric acid to pH 5.3. After stirring for 1 h in the cold room, the solution was centrifuged at 26 000 *g* for 20 min. The supernatant which contained LC1 was kept and the precipitate which mainly contained LC2 was dissolved in 10 m*M* Tris–HCl pH 7.5 and 50 m*M* NaCl with gentle stirring for 20 min. The LC2 fraction was further purified by reprecipitation at pH 5.3. This process could be repeated three or four times.

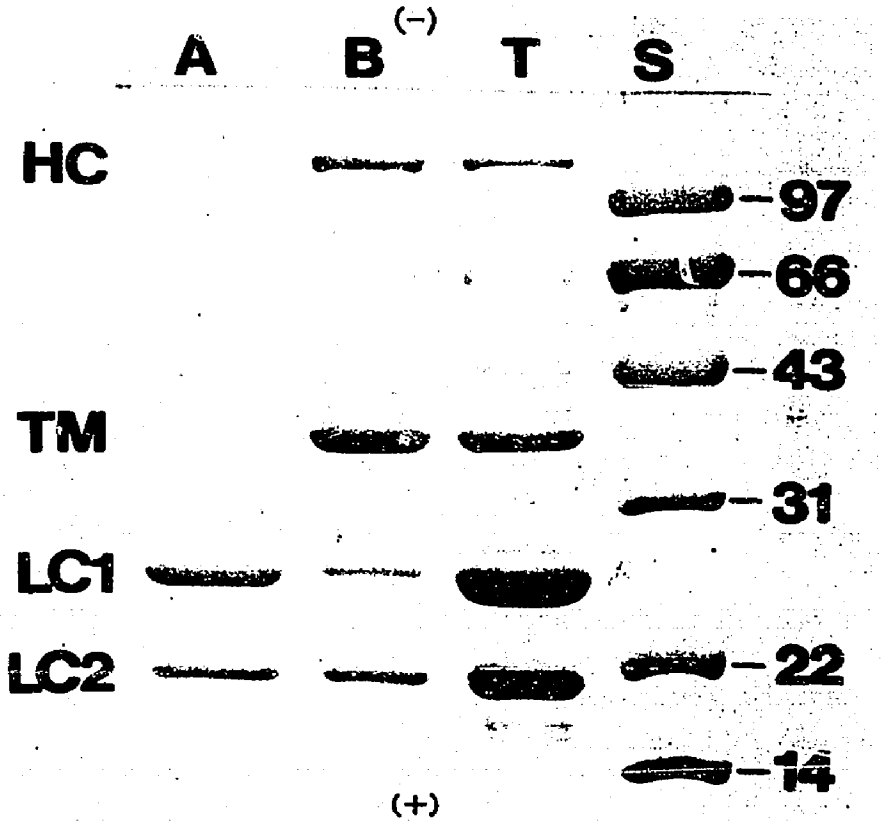

Fig. 1. 12% SDS-PAGE of human ventricular myosin light chains purified by isoelectric precipitation. Crude myosin light chains (T) were subjected to titration with 0.1 *M* citric acid to pH 5.3 in the presence of 10 m*M* $MgCl_2$. After centrifugation the main contaminating proteins, myosin heavy chain (HC) and tropomyosin (TM), were completely removed. Lanes: S = protein standard; A = supernatant after centrifugation; B = precipitate after centrifugation. A 10-μg amount of protein was used for lanes A and B, and a 20-μg amount of protein was used for lane T. The gel was stained with Coomassie Blue.

RESULTS

Preparation of myosin light chains

Electrophoretic analysis of the crude light chains (T in Figs. 1, 4 and 8) showed that light chains were contaminated mainly with myosin heavy chain and tropomyosin. We also noticed the appearance of two lower-molecular-mass components and variation in the quantities of LC1 and LC2. This variation has been previously observed by Klotz *et al.* [22] who suggested that it was the result of the specific degradation of LC2. When crude light chains were subjected to isoelectric precipitation by titration with 0.1 *M* citric acid to pH 5.3 in the presence of 10

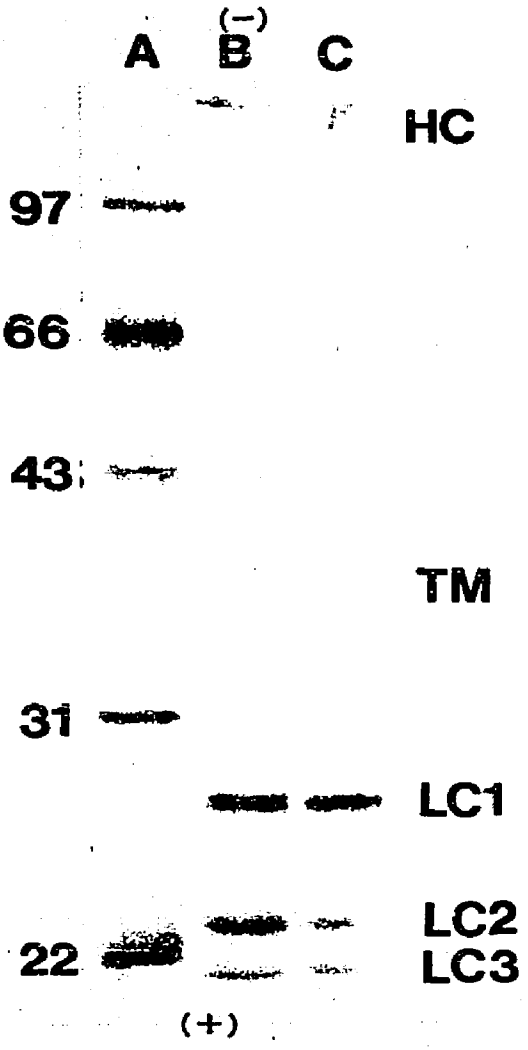

Fig. 2. 10% SDS-PAGE of myosin light chains from human gastrocnemius muscle purified by isoelectric precipitation in the presence of Mg^{2+}. Lanes: A = protein standard; B = myosin light chains before isoelectric precipitation; C = myosin light chains from supernatant after isoelectric precipitation in the presence of Mg^{2+}.

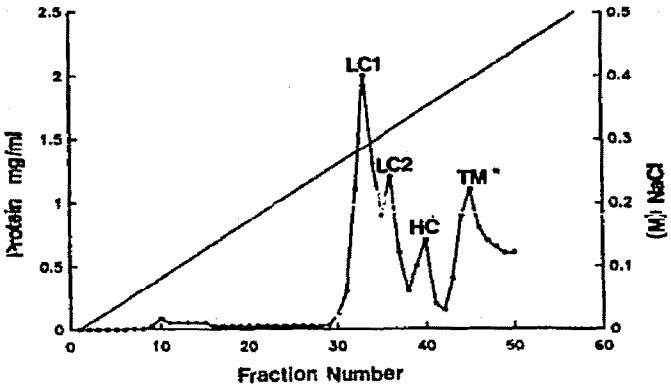

Fig. 3. Purification of myosin light chains from crude heart extracts by DEAE-Sephadex A-25. The light chains were eluted from the column by a 160-ml linear gradient of NaCl (0–0.7 *M*) in 0.05 *M* Tris–HCl pH 7.5 and 1 m*M* EDTA. Fractions of 2 ml were collected at a flow-rate of 20 ml/h. Protein concentrations were determined by the Bio-Rad protein assay method. Peaks: LC1 = light chain 1; LC2 = light chain 2; HC = heavy chain; TM* = tropomyosin and proteolytic fragments.

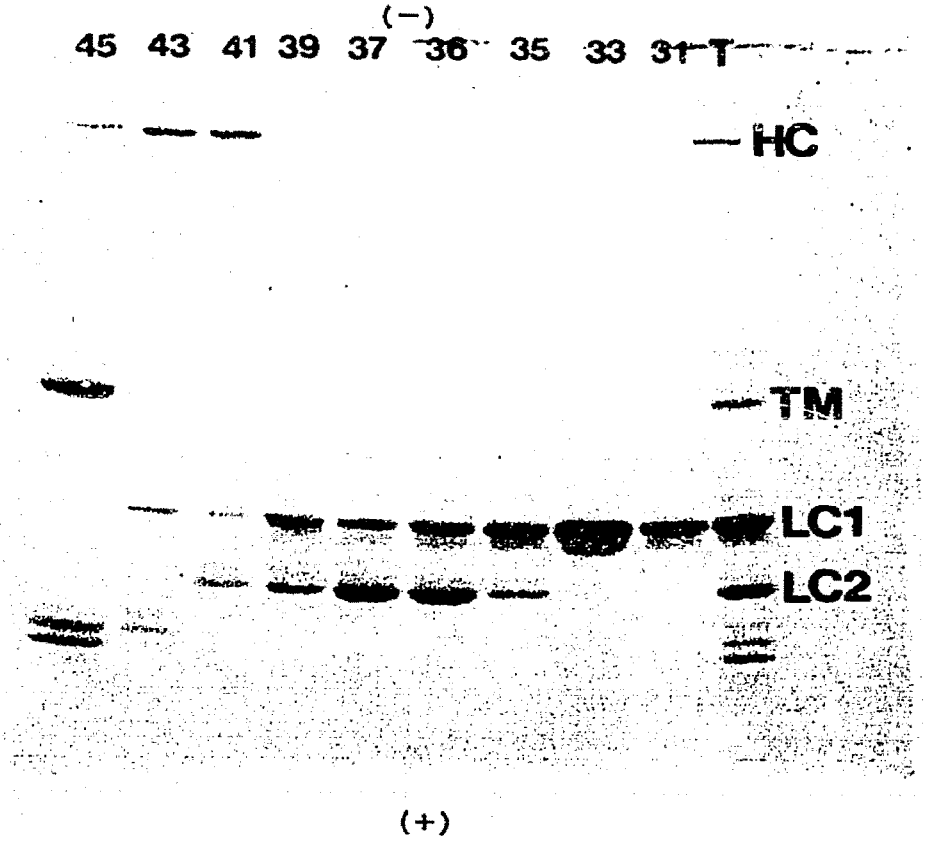

Fig. 4. 10% SDS-PAGE of the fractions selected in Fig. 3. T = Total crude human ventricular light chains; 31–45 = different fractions corresponding to the numbers indicated. LC1 was purified by the column, however, LC2 was contaminated with LC1.

m*M* $MgCl_2$, most contaminating proteins were removed leaving relatively pure light chains in the supernatant (A in Fig. 1). Similar results were obtained when this procedure was applied to the isolation of cardiac myosin light chains from twenty different animals (data not shown). Fig. 2 shows purified skeletal myosin light chains from human gastrocnemius muscle by this procedure.

Separation of myosin light chains by ion-exchange chromatography

Chromatography of the crude ventricular myosin light chains by DEAE-Sephadex A-25 is depicted in Fig. 3. Analysis of selected fractions from each peak by SDS-PAGE (Fig. 4) indicated that the proteins were eluted in order of LC1, LC2, HC, proteolytic products and tropomyosin. Part of the LC1 (31–33 in Fig. 4) was purified by chromatography, whereas LC2 was contaminated with LC1 (35–39 in Fig. 4). The protein band immediately below LC1 was proved to be the proteolytic fragment of LC1 by Western Blotting analysis using a monospecific antibody to human LC1. Similar results were obtained when pure preparations of human ventricular myosin light chains were used for chromatography (Fig. 5).

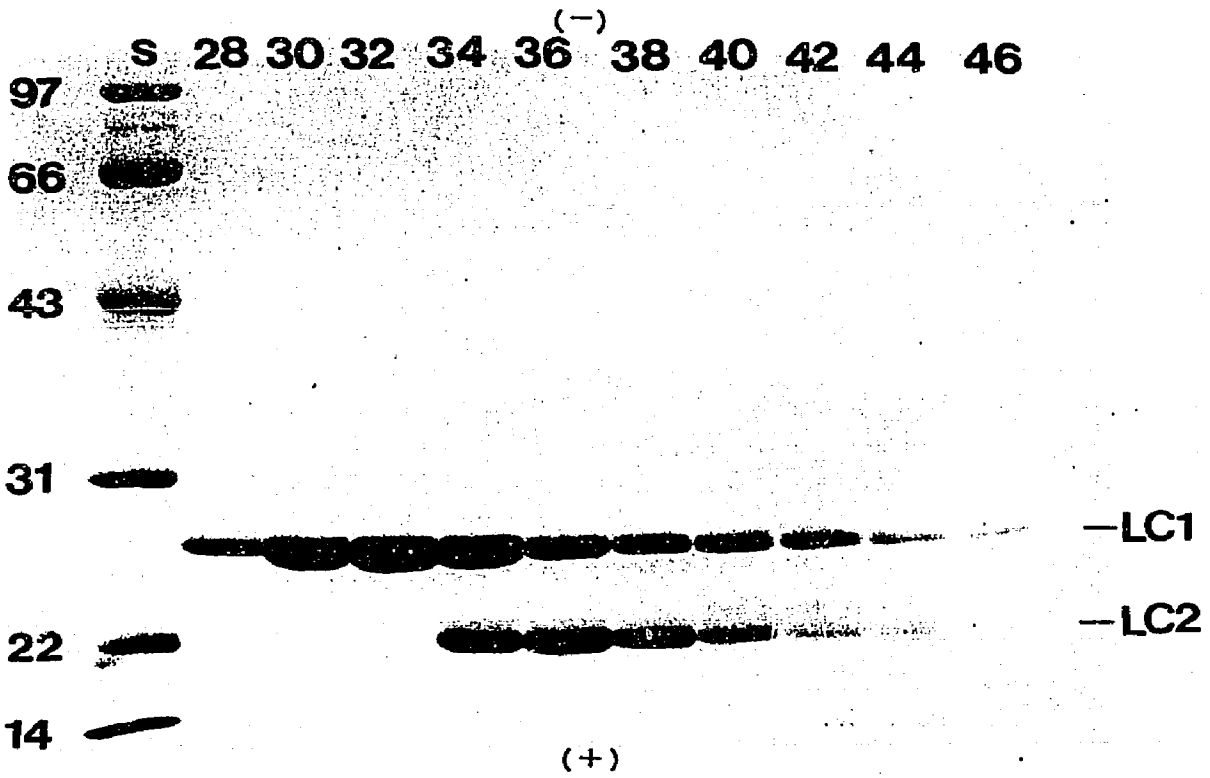

Fig. 5. 10% SDS-PAGE of fractions collected after ion-exchange chromatography of a pure preparation of human ventricular myosin light chains (A in Fig. 1). S = Protein standard; 28–46 = 50-μl samples from fractions with corresponding numbers. LC1 was highly purified (fractions 28–32). LC2 was contaminated with LC1 (fractions 34–46).

Purification of myosin light chains by reversed-phase HPLC

Myosin light chains purified either by ion-exchange chromatography (35–39 in Fig. 4) or by isoelectric precipitation in the presence of Mg^{2+} (A in Fig. 1) were separated into individual light chains by reversed-phase HPLC using a C_{18} column (Fig. 6). Two peaks were obtained and each peak was collected separately. Peaks 1 and 2 were proved to be LC1 and LC2, respectively, by SDS-PAGE (Fig. 6). The retention times for LC1 and LC2 were 14.2 and 15.4 min, respectively. HPLC analyses of each collected fraction (Fig. 6B and C) showed that both light chains were highly purified. We also attempted to use HPLC to directly purify individual myosin light chains from crude preparations. The results were not satisfactory because of inadequate separation of light chains from contaminating proteins (data not shown).

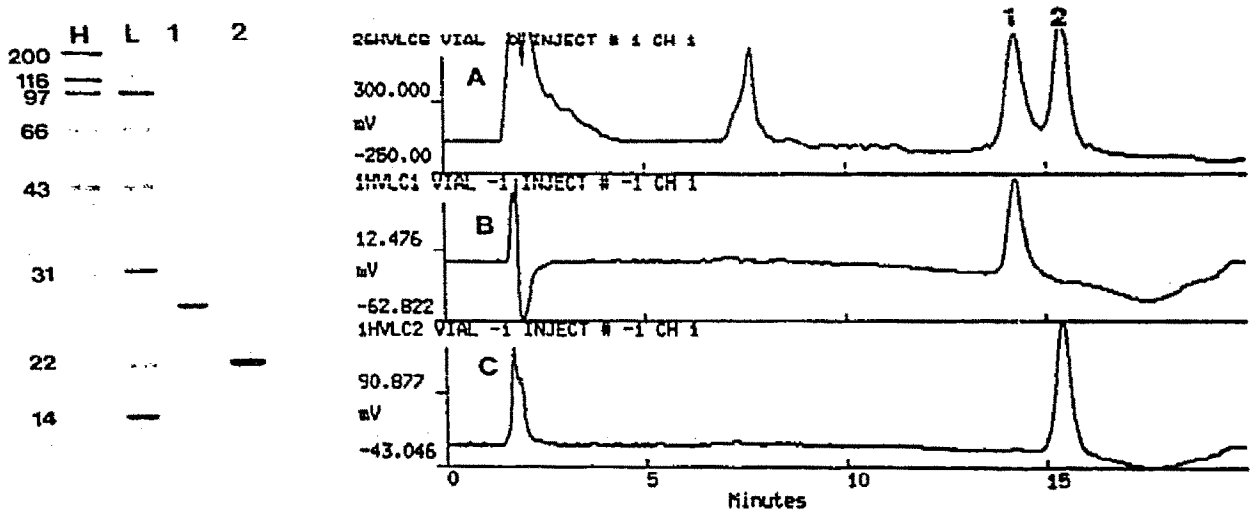

Fig. 6. Separation of myosin light chains by reversed-phase HPLC and electrophoretic analysis of the collected peaks. (A) Mixture of myosin light chains (peaks 1 and 2) pooled from fraction 34–40 in Fig. 5. (B) HPLC analysis of peak 1. (C) HPLC analysis of peak 2. 10% SDS-PAGE of the collected peaks is shown on the left side of HPLC profiles. Lanes: H and L = protein standard (Bio-Rad); 1 = peak 1; 2 = peak 2.

Separation of myosin light chains by isoelectric precipitation

Purified light chains were subjected to isoelectric precipitations by titration with 0.1 *M* citric acid to pH 5.3 in the absence of Mg^{2+}. Electrophoretic analyses of samples taken from both the supernatant and the precipitate after each centrifugation are shown in Fig. 7. The results indicated that, after the first precipitation step, the supernatant contained mainly LC1 (86%, based upon densitometric analysis). Traces of LC2 and other contaminants could be seen when the sample was overloaded (65 μg) (1 in Fig. 7), whereas the precipitate after the first centrifugation contained not only LC2 but also significant amounts of LC1 (48%) (4 in Fig. 7). After repeating the process three times on the precipitate, most of LC1

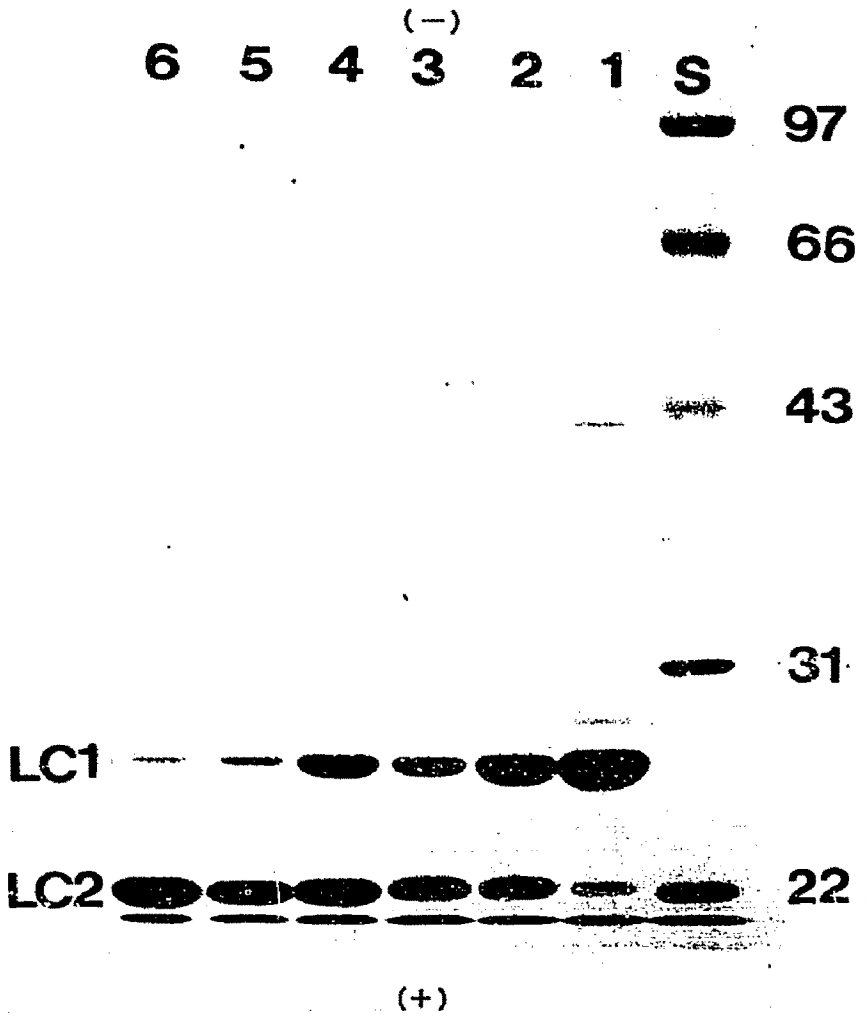

Fig. 7. 10% SDS-PAGE of samples taken from different steps during separation of individual myosin light chains from a pure preparation of human ventricular myosin light chains by isoelectric precipitation in the absence of Mg^{2+}. A 55–65 μg amount of protein was used for each well. Lanes: S = protein standard; 1–3 = supernatant after the first, second and third precipitation, respectively; 4–6 = precipitate from the first, second and third precipitation, respectively. Samples 1 and 6 are enriched in LC1 and LC2, respectively.

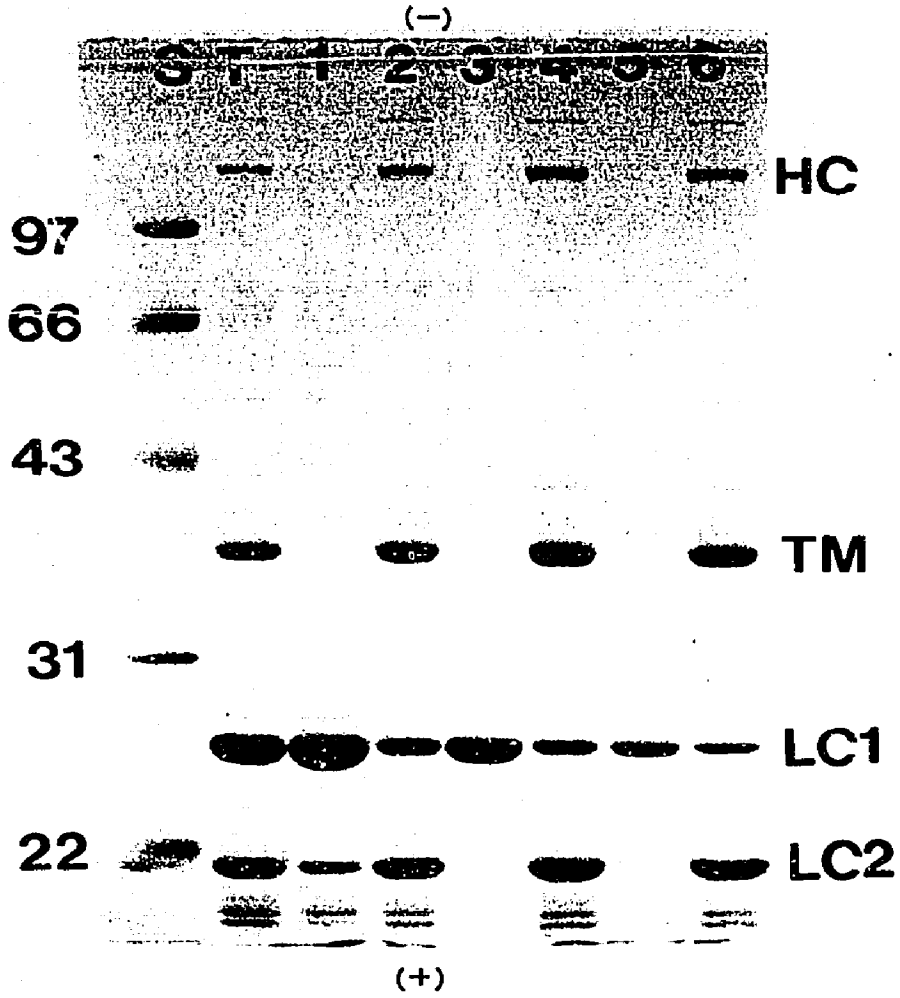

Fig. 8. 10% SDS-PAGE of samples taken from different steps in the separation of individual myosin light chains from a crude preparation of human ventricular myosin light chains by isoelectric precipitation in the absence of Mg^{2+}. Lanes: S = protein standard; T = crude light chains before separation; 1 and 2 = supernatant and precipitate, respectively, after the first precipitation; 3 and 4 = supernatant and precipitate, respectively, after the second precipitation; 5 and 6 = supernatant and precipitate, respectively, after the third precipitation. LC1 was roughly purified, however, contaminated proteins were always coprecipitated with LC2.

was removed from the LC2 fraction. Traces of LC1 (4%) still could be seen on SDS-PAGE when samples were overloaded (55 μg) (6 in Fig. 7). When crude light chains (T in Fig. 8) were subjected to isoelectric precipitation, the contaminating proteins including HC and tropomyosin were co-precipitated with LC2 (2, 4 and 6 in Fig. 8), but the supernatant contained relatively pure LC1.

DISCUSSION

In this report using the method of Wikman-Coffelt *et al.* [11] and our modification we were able to develop a rapid, easily performed procedure for isolating total cardiac myosin light chains. About 1.5–2 mg total cardiac myosin light chains per gram of heart material can be obtained by this method. The time needed from heart tissue to purify total myosin light chains is approximately three days. The isolation of mysoin light chains reported here was simplified by the elimination of the purification of myosin which ususally requires further purification by DEAE-Sephadex A-50 column chromatography [23] or DEAE-cellulose chromatography [10]. This simplification not only saves time but also increases the yield of myosin light chains. Although the main bulk of contaminating proteins, mainly HC and tropomyosin, can be easily eliminated by conventional isoelectric precipitation (Fig. 8), LC2 was removed at the same time (lanes 2, 4 and 6 in Fig. 8) since this method was used basically for the separation of individual myosin light chains from purified total myosin light chains. We discovered that in the presence of 10 m*M* Mg^{2+} myosin LC2, which normally was precipitated along with contaminating proteins, remained in the supernatant and only contaminated proteins were precipitated at acidic pH (Fig. 1). The supernatant containing total myosin light chains was dialyzed to remove Mg^{2+}, and then the individual light chain could be separated by conventional isoelectric precipitation. It was hypothesized that in the presence of Mg^{2+}, the phosphate groups of myosin LC2 may form complexes with Mg^{2+}, and these complexes in some way inhibit the formation of a precipitate of myosin LC2.

Comparing the amounts of LC1 and LC2 of purified myosin light chains (A in Fig. 1) showed that LC1 was always superior to LC2 in spite of the precaution of using fresh PMSF and keeping the temperature low throughout the preparation procedure. On the other hand, two lower-molecular-mass components were present (T in Figs. 1, 4 and 8) in front of LC2 on SDS-PAGE. The results of our Western Blotting analyses using our LC2 monospecific antibody showed that these two components had antigenicity with LC2 (data not shown). Similar lower-molecular-mass components have been observed previously and proved to be proteolysis of LC2 [22]. The instability of human myosin LC2 might explain why the amount of LC1 was always superior to that of LC2. This modified procedure for the isolation and purification of cardiac myosin light chains has been successfully applied to twenty different animals (data not shown). It seems also suitable for the purification of skeletal myosin light chains: human skeletal myosin light chains have been isolated and purified by this procedure in our laboratory.

DEAE-Sephadex A-25 has been employed for a long time for the separation of myosin light chains [8,10]. Only crude myosin is needed for this method resulting in a higher yield of myosin light chains. It is an easy and effective procedure, specifically for the purification of alkali myosin LC1, although the separation of the two light chains is not adequate in our experiment. The same result was

obtained previously [24]. Myosin LC2 fractions were always contaminated with varying amounts of LC1 depending on their ratio before separation. Since LC1 was always superior to LC2 in the preparation of myosin light chains from human ventricle, human LC2 fractions after column chromatography were contaminated with significant amounts of LC1. The buffer system containing KCl and urea as described earlier [10] was originally employed in our laboratory. Since KCl severely affects the mobility of proteins when fractions are analyzed directly by SDS-PAGE, we replaced KCl by NaCl which does not affect the retardation coefficients, free mobilities and R_F values for proteins (M_r 20 000–66 000 up to 0.8 M [25]. To further simplify this procedure, chromatography was performed at room temperature without using urea. No significant decrease was observed in the yield of myosin light chains.

Separation of myosin light chains by reversed-phase HPLC gave high purity of individual light chains. Only 20 min were needed for each run. A relatively pure preparation of myosin light chains was required for HPLC because contaminants could not be removed by this method. Pure preparations of myosin light chains can be obtained from crude ones either by DEAE-Sephadex A-25 chromatography described earlier or by isoelectric precipitation in the presence of Mg^{2+}.

Separation of myosin light chains by isoelectric precipitation results in the highest yield among the three procedures. Although the separation was not satisfactory, this procedure does not require expensive chemicals and sophisticated equipment and is easy to perform.

Ion-exchange chromatography is recommended for the purification of cardiac myosin LC1, because it is a relatively simple method and requires only crude preparations of myosin light chains before separation. Isoelectric precipitation could also be employed if high yield is more important than high purity. For the purification of myosin LC2, HPLC is the only method which gives high purity. Isoelectric precipitation can only be used if high purity of LC2 is not required.

All results presented here are based on the isolation and separation of human ventricular myosin light chains. The results obtained from ventricles of other species or from skeletal muscle may vary to a certain degree. For example, cardiac myosin light chains isolated from sheep ventricle did not show degradation of LC2 [26] and also the contamination of LC2 fractions with LC1 after ion-exchange chromatography was much less.

In summary, the procedures we have described are not only useful for human muscle tissue but can also be applied successfully to many different species.

ACKNOWLEDGEMENTS

We would like to thank Dr. Barry R. Blenkinsop, Dr. John H. Smith and Dr. Ross C. Bennett from the Forensic Pathology Branch, (Ontario, Canada) for obtaining human hearts. This work was supported by Grant MA 8559 from the Medical Research Council of Canada. G. J. is a scholar of the Medical Research Council of Canada.

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