MYOSIN CHAINS OF MYOCARDIAL TISSUE - I. PURIFICATION AND IMMUNOLOGICAL PROPERTIES OF MYOSIN HEAVY CHAINS[1]

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Summary:

An antiserum specific to dog myocardial myosin has been developed against highly purified myosin heavy chains. The antiserum is specific for the heavy chains of myosin, giving a single precipitin line in an immunodiffusion assay for either the heavy chains of myosin or native myosin, and does not react with any other myocardial proteins. In such assays myosin acts as a single, uniform antigen. Using this antiserum, a radioimmunoassay has been developed to quantitate myosin in a homogenate of myocardial tissue containing free myosin dissociated from other cellular components.

Materials and Methods:

Purification of Myocardial Myosin Heavy Chains. The hearts were removed from the dogs, fat and vessels dissected away, and the hearts immersed in liquid nitrogen and stored frozen (-70°C) (1). All subsequent steps were carried out at 4°C, except where indicated. The tissue was minced and then sheared 10 times, 10 sec each (Sorvall Omni-mixer, 40,000 rpm) in 2.5 vol of 0.05 M PO_4 buffer, pH 6.8 (2) containing 0.001 M EDTA (2), 0.01 M Na pyrophosphate (3), and 0.001 M DTT*(4). The added pyrophosphate in the wash and subsequent extractions helped reduce protein interaction and increase yield. The minced tissue was centrifuged for 5 min at 10,000 x g and the pellet obtained was sheared for the same period of time in 3 vol of 0.3 M KCl (2), 0.1 M KH_2PO_4, 0.05 M K_2HPO_4, 0.01 M Na pyrophosphate, 0.001 M DTT, 0.001 M EDTA. The homogenate was stirred for 10 min and then centrifuged

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*Abbreviation: DTT, dithiothreitol

1,300 x g for 10 min. (A short extraction period reduced solubilization of actin (2)). The supernatant was removed and again centrifuged 10,000 x g for 5 min. The supernatant was filtered through 2 layers of cheesecloth. Myosin crystals were obtained by adding 9 vol of water containing 0.001 M EDTA (2), stirring for 10 min and then centrifuging at 10,000 x g for 10 min. The pellet was dissolved (2 mg of protein/ml) by homogenizing in a teflon-glass homogenizer (3 strokes) in 0.01 M Tris buffer, pH 7.5 containing 0.5 M KCl, 0.001 M EDTA, 0.005 M $MgCl_2$ and 0.001 M DTT. After dissolving, it was made 0.002 M ATP. The solution was stirred for 10 min and then centrifuged at 100,000 x g for 1 hr. This step removed remaining free actin and other contaminants (5). The supernatant was adjusted to pH 6.6 by adding solid $NaHCO_3$ and diluting the KCl to 0.3 M. After stirring for 10 min at room temperature (2) the actomyosin remaining was precipitated by then centrifuging at 10,000 x g for 10 min. The supernatant was stirred for 15 min with DEAE Sephadex A-25 (1) which had been washed, charged and equilibrated with 0.2 M KCl (6,7), and 0.1 M Tris, pH 7.6 . The Sephadex comprised 30% of the volume. The Sephadex was removed by suction filtration through a Buchner funnel. After removal of nucleotides, the filtrate was adjusted to 0.3 M KCl by adding 3 M KCl and to pH 6.6 by adding 1 M KH_2PO_4 (2). The solution was diluted with 7 vol of water containing 0.001 M EDTA, stirred for 30 min and then centrifuged 10,000 x g for 15 min. The pellet was suspended (2 mg of protein/ml) in 0.05 M pyrophosphate buffer, pH 7.5 containing 0.001 M EDTA and 0.001 M DTT. After dissolving, the ATP was added to 0.002 M and stirred for 10 min. This buffer system kept myosin dissociated from other cellular proteins so it could be specifically precipitated. Using saturated $(NH_4)_2SO_4$ adjusted to pH 6.8 (8) the fraction between 35% and 45% saturation with $(NH_4)_2SO_4$, was collected and the pellet dissolved by homogenizing in 0.05 M Tris, pH 7.5, 0.001 M DTT and 0.5 M KCl. The myosin was precipitated with 10 vol of water containing 0.001 M EDTA, centrifuged and solubilized again in the above buffer for $(NH_4)_2SO_4$ precipitation, and the fraction be-

tween 37.5% and 42.5% saturation with $(NH_4)_2SO_4$ was collected as pure myosin. (The $(NH_4)_2SO_4$ saturation precipitation point for dog myocardial myosin is lower than that for skeletal muscle myosin (8)). At salt concentrations higher than 42.5% $(NH_4)_2SO_4$ saturation, tropomyosin (9) precipitated as a contaminant protein. Myosin was dissolved in 0.05 M Tris, pH 7.5, 0.001 M DTT and 0.5 M KCl and dialyzed overnight against the same buffer. The myosin was precipitated by adding 10 vol of water containing 0.001 M EDTA. (Dilutions with water precipitates myosin and helps solubilize contaminant proteins (9,10,11).

For obtaining the heavy chains the myosin pellets were dissolved in 0.1 M KCl, 0.001 M DTT, 2.0 M LiCl, 0.1 M glycine, pH 11.35 (12,13), and after dissolving made 0.002 M ATP. The myosin solution was stirred for 30 min at 4°C. The heavy chains were precipitated with 2.5 M K citrate, pH 7.0 (12,13). After stirring for 10 min, the dissociated complex was centrifuged at 12,000 x g for 15 min. In order to completely remove all light chains from myocardial myosin, as reported here, further steps for complete removal of light chains were necessary. The pellet was suspended in 8.0 M urea, 0.001 M DTT, 0.01 M EDTA, and 0.05 M Tris, pH 7.5. (Lower concentrations of urea as used for dissociation of skeletal muscle myosin chains (13) did not completely remove all myocardial myosin light chains). After stirring for 2 hrs at room temperature the heavy chains were precipitated by adding 10 vol of water, stirring for 10 min and centrifuging 20,000 x g for 10 min. Finally, in order to remove any contaminants which precipitated in the 37.5% to 42.5% $(NH_4)_2SO_4$ saturation fraction, the heavy chains from several purifications were pooled, dissolved in 0.5 M KCl, 0.01 M Tris, pH 7.5 and 0.001 M EDTA and were again subfractionated. The myosin heavy chains, which precipitated at a lower $(NH_4)_2SO_4$ fractionation than whole myosin, were precipitated between 0 and 34% saturation with $(NH_4)_2SO_4$.

Prepartion of Antiserum. Antiserum was prepared as described earlier (14-17). For preparation of antiserum 0.5 ml of myosin heavy chains containing 1 mg of protein was mixed with an equal vol of Freund's adjuvant and

injected into the axilla of goats. The first 2 injections were 14 days apart, followed by 3 injections every 7 days. After 5 injections the goats were bled and continued on monthly injections of antigen.

Immunodiffusion Assays. For immunodiffusion plates, 1% agarose was mixed with 0.05 M PO_4 buffer, pH 7.5, containing 0.3 M KCl, 0.1% thimerosal and 0.001 M EDTA. Plates were prepared and treated as described earlier (16).

Radioimmunoassay for Myosin. For radioimmunoassays the purified myosin heavy chains (HC) were labeled with [^{125}I] as described earlier for ribosomal proteins (16). Myosin (HC) (labeled and unlabeled) was dissolved and dialyzed in the immunoassay buffer which was 0.05 M PO_4, pH 7.5, containing 0.3 M KCl, 0.001 M EDTA, 10% glycerol and 0.1% albumin. A 0-40% $(NH_4)_2SO_4$ precipitation was made on the goat anti-myosin (HC) serum, the goat serum and the second antibody, rabbit anti-goat-γG serum. The partially purified γG was dialyzed against the immunoassay buffer, as above, but without glycerol and albumin. The total incubation vol which was 0.3 ml consisted of 0.1 ml [^{125}I]-myosin (HC) (50,000 cpm), 0.1 ml (10 μg) of γG of goat anti-myosin (HC), and 0.1 ml of either buffer, unlabeled myosin heavy chains, or unlabeled native myosin. After 1 hr incubation at 37°C, 0.1 ml (200 μg) of goat γG was added as carrier and 0.1 ml (1 mg) of the second antibody (rabbit anti-goat-γG) was added. Incubation was continued at 37°C for 1 hr and then centrifuged at 10,000 x g for 10 min and the pellet counted in a gamma counter to determine antibody-bound myosin as described earlier (14,16,17).

Preparation of Homogenate for Myosin Determination. For quantification of myosin, 0.5 gm of myocardial tissue was minced and sheared (10 times, 10 sec each, 30,000 rpm) using a micro-attachment for the Sorvall Omni-Mixer, in 4 vol of 0.05 M PO_4 buffer, pH 6.8 containing 0.001 M EDTA and 0.25 M sucrose. The sheared tissue was centrifuged at 10,000 x g and the pellet obtained was again sheared (3 times, 20 sec each, 30,000 rpm) in the same vol of buffer containing 0.3 M KCl, 0.1 M KH_2PO_4, 0.05 M K_2HPO_4, 0.01 M Na pyrophosphate, 0.001 M EDTA and 0.001 M DTT. The homogenate was stirred for 10 min at 4°C and a sample removed for DNA analyses (14,16). The remaining homogenate was centrifuged 40,000 x g, 10 min. The supernatant was

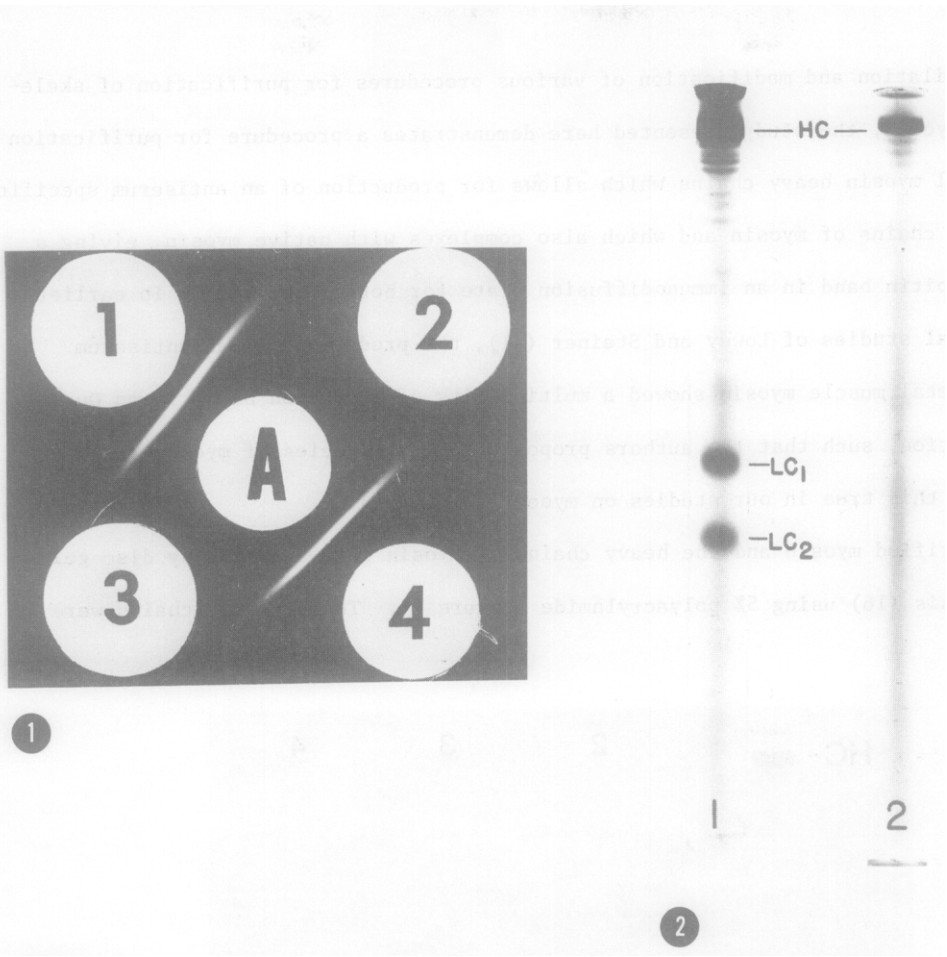

Figure 1
Gel electrophoresis of (1) purified myosin and (2) the heavy chains of myosin. (HC) denotes the heavy chains of myosin; (LC_1) and (LC_2) are the two light chains of myosin.

Figure 2
Gel electrophoresis of the purified chains of myosin. (1) heavy chains of myosin; (2) the two light chains of myosin; and (3&4) further purification of the two light chains of myosin.

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diluted 5 fold with the above buffer, made 0.002 M ATP and stirred for 5 min at room temperature. After centrifuging at 40,000 x g for 10 min, the homogenate was immediately used to compete out [^{125}I]-myosin (HC) in a radioimmunoassay.

Results and Discussion:

Earlier reports on purification of myocardial myosin (1,18,19,20),like skeletal muscle myosin (2,3,6,8), did not demonstrate, in our laboratory, a degree of protein purity of dog myocardial myosin sufficient for myosin-specific antibody production.

Using a compilation and modification of various procedures for purification of skeletal muscle myosin, the study presented here demonstrates a procedure for purification of myocardial myosin heavy chains which allows for production of an antiserum specific to the heavy chains of myosin and which also complexes with native myosin, giving a single precipitin band in an immunodiffusion plate for both (Figure 1). In earlier immunochemical studies of Lowey and Steiner (21), the production of an antiserum against skeletal muscle myosin showed a multiplicity of diffusion bands in an Ouchterlony reaction, such that the authors proposed several species of myosin (21). We did not find this true in our studies on myocardial myosin.

The purified myosin and the heavy chains of myosin were analyzed by disc gel electrophoresis (16) using 5% polyacrylamide (Figure 2). The purified chains were

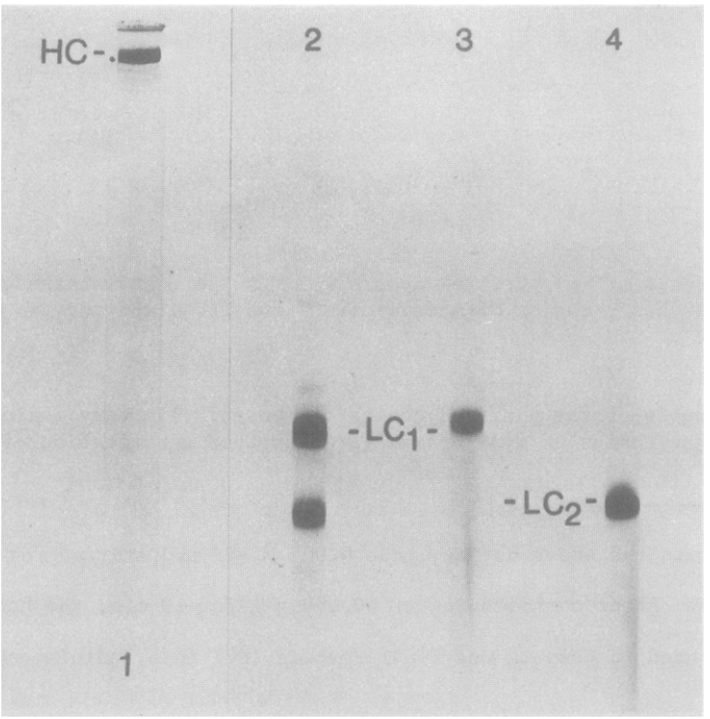

Figure 3
Immunodiffusion pattern of native myosin, heavy chains of myosin, the two light chains of myosin, and antiserum to the heavy chains. (1) native myosin; (2) first myosin light chain (LC_1); (3) second light chain of myosin (LC_2); the heavy chains of myosin (HC); and (A) antiserum to the heavy chains of myosin.

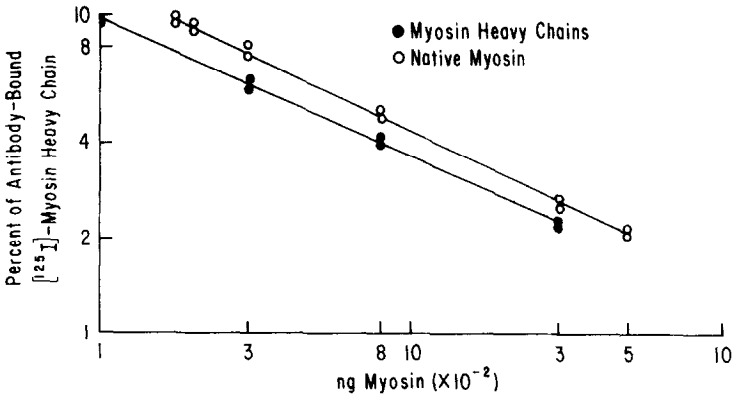

Figure 4
Radioimmunoassay of the heavy chains of myosin, native myosin and antiserum to the heavy chains. Competition of heavy chains of myosin against [^{125}I]-myosin heavy chains (●—●) and competition of native myosin against [^{125}I]-heavy chains (○—○).

analyzed on 7% polyacrylamide (Figure 3). The 2 light chains or light subunits are located in the HMM S-1 heads (13) and are dissociated from the remaining myosin molecule, i.e., the 2 heavy chains or heavy subunits, by the procedure used here. Based on the position of standards, the heavy chains (Figure 2,2;3,1) had a molecular weight of 210,000; the two light chains had a molecular weight of 30,000 (Figure 3,2&3) (LC_1) and 20,000 (Figure 3,2&4) (LC_2). In further studies each of the 2 light chains were purified separately and were shown to again complex with the heavy chains in vitro (22). Furthermore, 100% of the myosin ATPase activity could be reestablished after reconstitution of the myosin complex, both chains being necessary to regain total ATPase activity (22).

Both myosin heavy chains and native myosin (Figure 4) were shown to compete out [^{125}I]-myosin (HC). The variation in concentration of heavy chains and native myosin for competition against [^{125}I] heavy chains is due to (a) the additional weight of the light chains in native myosin, where protein concentration is based on Lowry determinations (23), and (b) where all the determinant sites are present on a single antigen as in native myosin, as compared to the equivalence of 2 antigens containing the determinant sites with the heavy chains. Such differences were also found in competition of ribosomes for antiserum against the 60S ribosomal subunits (15). Thus, using the antiserum to the heavy chains of myosin, native myosin could

be quantitated and specifically precipitated from a homogenate, prepared as described here, where myosin was kept in a free form dissociated from other myocardial components.

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