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ELECTROPHORETIC AND IMMUNOLOGICAL STUDIES ON THE LIGHT CHAINS OF MYOSIN

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Myosin was highly purified mainly by combining procedures of ammonium sulfate fractionation and chromatographies on cellulose phosphate, DEAE-cellulose and DEAE-Sephadex A-50 as judged by gel electrophoresis and immunological analyses.

The highly purified myosin produced two precipitin lines in immunodiffusion with antibodies to myosin, whereas the myosin produced only one peak in quantitative precipitin analysis. This difference implied the possibility that agar gel promoted the dissociation of light chains from myosin.

Immunological analyses of light chains prepared from the myosin showed the presence of two kinds of light chain components. On the other hand, light chains were separated usually into two components, L-1 (molecular weight 27,000) and L-2 (mol. wt. 17,000) in SDS-gel electrophoresis. However, an additional component, L-3 (mol. wt. 14,000) was sometimes appeared in the electrophoresis of light chains. The possibility that L-3 was a breakdown product of L-1 was discussed.

INTRODUCTION

Myosin is contractile protein of striated muscle and has ATPase and actin binding activities.

In denaturating solvents the myosin molecule dissociates into several polypeptide chains; two heavy chains (mol. wt. 190,000~210,000) and two to four light chains (mol. wt. 20,000~30,000)[5~7]. The light chains are thought to be localized in subfragment-1[20], biologically active fragment produced by enzymic digestion, and to be essential to ATPase and actin binding activities[1,2,4,19].

There exists, however, some controversy concerning the number and the kinds of light chains. Light chains were separated to two to four bands in SDS-gel electrophoresis[15,16,18]. However, the purification of myosin has not yet been perfected, and the possibility exists that some components of the light chain preparation are tightly bound contaminants.

Light chains are separated from myosin mainly by alkali treatment (above

pH 10.5). This treatment may cause a hydrolytic cleavage of peptide bonds which yields an incorrect number of polypeptide chains.

To clarify the ambiguities, we have attempted the purification of myosin and the preparation of light chains from purified myosin without a cleavage of peptide bonds. We have introduced immunological methods to the assay on the purity of myosin and the determination of the number and kinds of light chains.

Materials and methods

Preparation of myosin Myosin was prepared from chicken superficial pectoral muscle by extracting the mince for 7 min with 3 volumes of a solution consisting of 0.3 M KCl, 0.09 M KH_2PO_4, 0.06 M K_2HPO_4, 1 mM EDTA and 0.25 mM ATP (pH 6.4, I=0.55). The extract was separated from the residues by centrifugation at 600 ×g for 10 min, and was then diluted with water to precipitate myosin. The precipitate was dissolved in 0.5 M KCl, 20 mM Tris-HCl buffer (pH 7.5) and fractionated with $(NH_4)_2SO_4$ between 37 and 42% saturation. After dialysis against 0.25 M KCl, 20 mM Tris-HCl (pH 7.5), the myosin was centrifuged at 107,000 ×g for 60 min to remove any actomyosin. The supernatant (15~20 mg/ml) was diluted to 3~4 mg/ml with 0.25 M KCl, 20 mM Tris-HCl (pH 7.5) and then was passed first through a cellulose phosphate column and subsequently through a DEAE-cellulose column, according to the procedure of Harris & Suelter. The myosin at this step was used as immunogen for rabbits. The myosin was further purified by chromatography on DEAE-Sephadex A-50 according to the method of Richards et al.

Preparation of light chains Light chains were prepared essentially by the method of Perrie & Perry. Myosin (10~20 mg/ml) was exposed to 5 M guanidine-HCl and 10 mM 2-mercaptoethanol and stirred overnight. The solution was diluted with an equal volume of water and subsequently with four volumes of ethanol, and then centrifuged at 5,000 ×g for 10 min to precipitate heavy chains. Ethanol in the supernatant was evacuated under reduced pressure and the resulting solution was dialyzed against 20 mM Tris-HCl (pH 7.5). The light chains obtained (0.2~0.3 mg/ml) were concentrated by chromatography on DEAE-cellulose. By one-step elution with 0.4 M NaCl, 20 mM Tris-HCl (pH 7.5), 80 to 90% of applied proteins were collected. The light chains were fractionated with $(NH_4)_2SO_4$ between 30 and 70% saturation.

Heavy chains precipitated by ethanol were dialyzed against water and

Abbreviations: SDS, sodium dodecyl sulfate; anti-myosin, rabbit antiserum (or γ-globulin-enriched fraction) to chicken myosin; anti-light chains, rabbit antiserum (or γ-globulin-enriched fraction) to chicken light chains

dissolved in 20 mM sodium phosphate buffer (pH 7.0) containing 5 M urea.

Protein determination Protein concentration was determined by optical density measurement at 280 nm based on micro-Kjeldahl analysis, unless otherwise stated. The following values for $A_{280nm}^{1\%}$ were used: 5.25 (myosin) and 4.03 (light chains).

Gel electrophoresis Electrophoresis of sulfonated myosin and heavy chains were performed by the method of PATERSON & STROHMAN. Myosin was incubated at 27°C for 2 hr in 0.1 M sodium sulfite, 0.1 M Tris-HCl (pH 8.5), 0.05% SDS, 0.05% 2-mercaptoethanol and 12.5% glycerol at a protein concentration of 2~3 mg/ml. Heavy chains were sulfonated in this solution in the presence of 2.5 M urea. Electrophoresis of sulfonated proteins were performed in a 25 mM Tris-glycine buffer (pH 8.8), 0.05% SDS and 10% glycerol on 3.36% polyacrylamide gels at a 1.5 mA/tube for 1.5~2 hr. The gels were stained in 0.1% Amido Black-20% acetic acid and destained electrophoretically.

SDS-gel electrophoresis of light chains was carried out according to the method of WEBER & OSBORN. Light chains were incubated at 37°C for 2 hr in 10 mM sodium phosphate (pH 7.0) containing 8 M urea, 1% SDS and 1% 2-mercaptoethanol. The protein solution was dialyzed against 10 mM sodium phosphate (pH 7.0) containing 0.1% SDS and 0.1% 2-mercaptoethanol overnight at room temperature. Electrophoresis was performed in 10% polyacrylamide gels at an 8 mA/tube for 4~5 hr. Coomassie brilliant blue was used for staining by the method of FAIRBANKS et al. Molecular weights were determined with the use of a calibration curve obtained by plotting the electrophoretic mobilities of various highly purified proteins (Mann) against the logarithm of their known polypeptide chain molecular weights.

Preparation of antisera Myosin and light chains (10 mg, respectively) in 0.3 M NaCl, 20 mM sodium phosphate (pH 7.0) emulsified with an equal volume of Freund's complete adjuvant (Difco) were injected subcutaneously into rabbits at 1 week intervals. Four injections were required for obtaining a high titer of antisera. The rabbit were bled 8~10 days after the last injection from the carotid artery. Sometimes, antigens were injected directly into popliteal lymph node so as to maintain a high titer of antisera. γ-Globulin-enriched fractions were obtained by repeating fractionation with $(NH_4)_2SO_4$ between 25 and 40% saturation. The γ-globulin fractions were lyophilized and stored at −25°C until use.

Immunodiffusion analysis Immunodiffusion was carried out by the method of Ouchterlony with 1% agar (Wako, special agar B) in 20 mM sodium phosphate (pH 7.0) containing 0.3 M NaCl for myosin and 0.15 M NaCl for light chains;

10 ml of agar were placed in a Petri dish 8.5 cm in diameter. The diffusion plates were incubated for 24 hr at room temperature and then allowed to settle at 4°C for 6 days. The plates were photographed by dark field illumination. Immunodiffusion with agarose (1 %) in stead of agar was also carried out as described above.

Quantitative precipitin analysis Antigens were allowed to react with antibodies in 20 mM sodium phosphate (pH 7.0) containing 0.5 M NaCl for myosin and 0.15 M NaCl for light chains. In each case, the reaction mixture consisted of 0.2 ml antigens in varying concentrations and 0.2 ml γ-globulin (2 mg). The mixtures were incubated at 37°C for 60 min and then were allowed to stand at 4°C for 4 days. After 3 washes with each salt solution, the immunoprecipitate was analyzed for protein by the method of LOWRY et al.

RESULTS

The purity of myosin

The purity of myosin was assayed by immunodiffusion and quantitative precipitin analysis with homologous antibodies. In immunodiffusion (fig. 1), three or more precipitin lines were produced by myosin before chromatography on DEAE-Sephadex A-50. After the chromatography, the myosin produced only two lines with anti-myosin. The inner line, located near the antibody well, fused with the line produced by light chains and anti-myosin, indicating that the inner line corresponded to the light chains. On the other hand, when sulfonated myosin reacted with anti-myosin instead of the untreated myosin, the outer line disappeared and a straight line was produced closer to the antibody well (fig. 2). This fact suggests that the outer line corresponds to two heavy chains, which are dissociated to single heavy chain by sulfonation, and that its molecular weight is of the same magnitude as anti-myosin γ-globulin[11]. The line corresponding to the light chains that was produced by sulfonated myosin and anti-myosin, completely fused with the inner line produced by the untreated myosin and anti-myosin. The production of two lines by myosin and anti-myosin occurred in immunodiffusion by using agarose gel instead of agar. These results revealed that light chains were separated from myosin in immunodiffusion and are in agreement with those of other investigators[9,10,12].

In quantitative precipitin analysis, only one peak was produced by myosin after chromatography on DEAE-Sephadex A-50 and anti-myosin. On the other hand, the myosin before chromatography showed one peak with a broad shoulder (fig. 3). These experiments indicated that immunologically purified myosin was obtained after the step of DEAE-Sephadex A-50 chromatography. This purified myosin was used in later experiments.

FIGURE 1
Immunodiffusion of myosin and light chains with anti-myosin

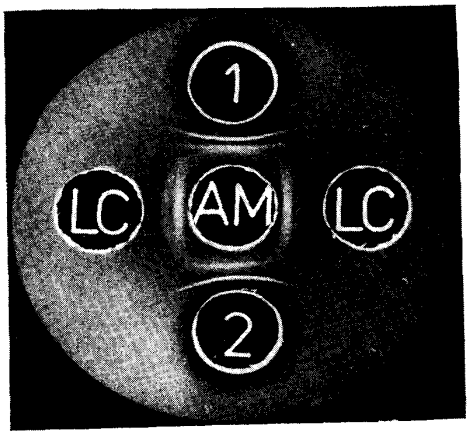
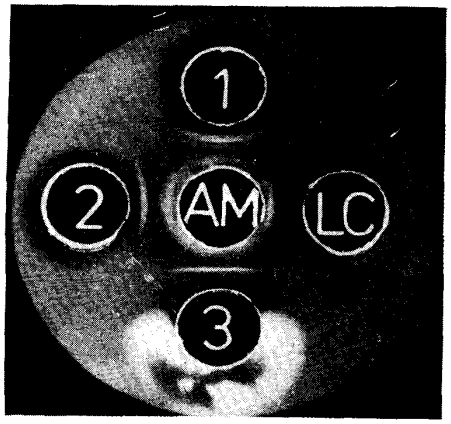
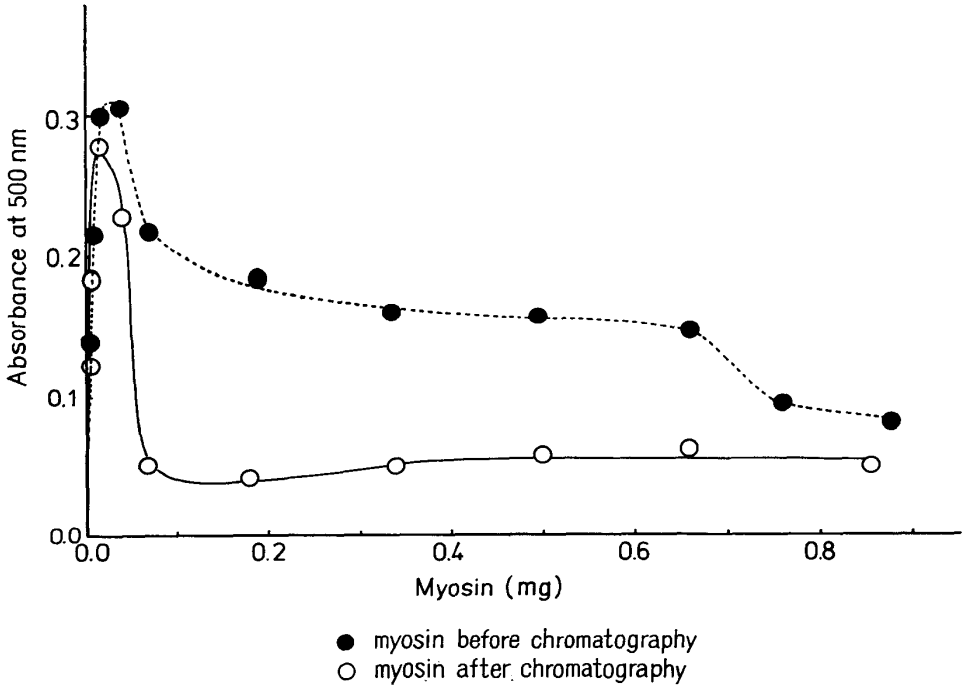


FIGURE 2
Immunodiffusion of untreated and sulfonated myosin with anti-myosin



1: myosin before chromatography (4 mg/ml), 2: myosin after chromatography (4 mg/ml), 3: sulfonated myosin (4 mg/ml), LC: light chains (0.04 mg/ml), AM: anti-myosin (undiluted serum)

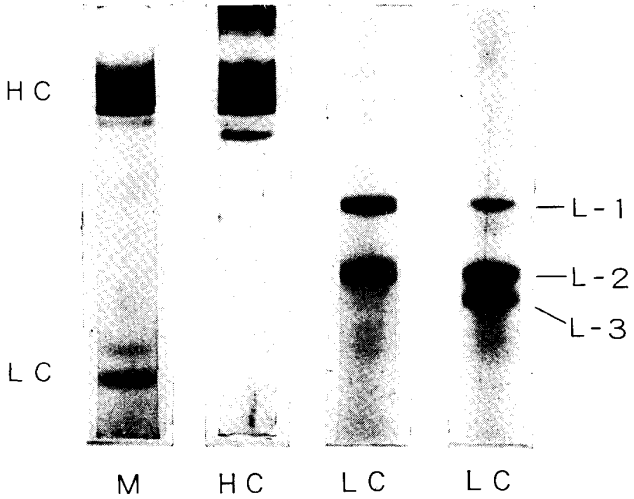
FIGURE 3 *Quantitative precipitin analyses of myosins and anti-myosin*



Gel electrophoresis and the immunological analysis of light chains

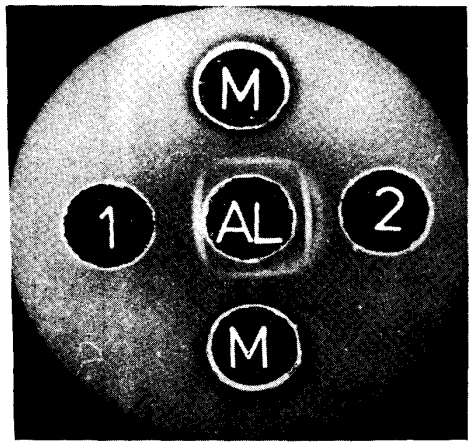
The yield of light chains was 9 to 10% of myosin after the step of DEAE-cellulose chromatography in the preparation procedures of light chains. The gel

FIGURE 4
Gel electrophoresis of myosin, heavy chains and light chains



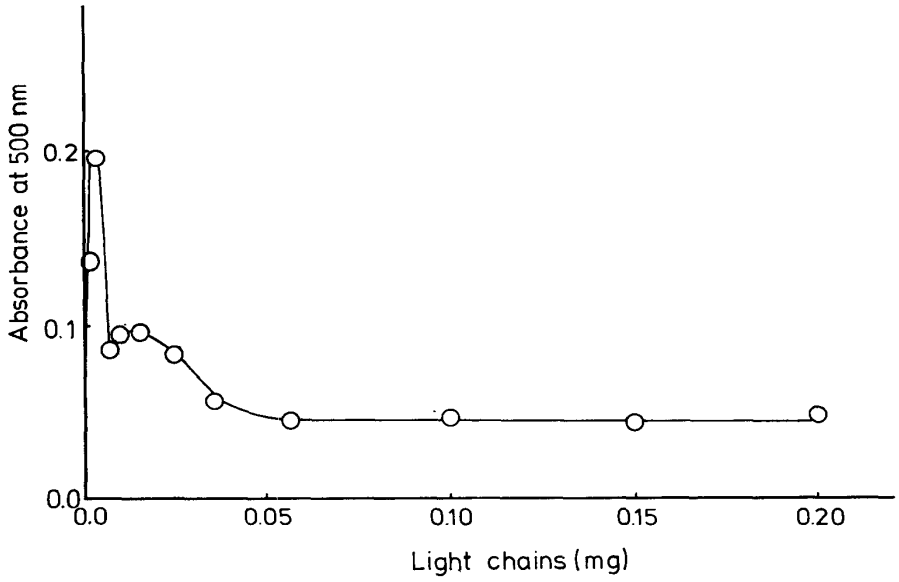
M: myosin (100 μg), HC: heavy chains (100 μg), LC: light chains (20 μg)

FIGURE 5
Immunodiffusion of light chains and anti-light chains



1: light chains (0.4 mg/ml), 2: light chains (0.04 mg/ml), M: myosin (4 mg/ml), AL: anti-light chains(undiluted serum)

FIGURE 6 *Quantitative precipitin analysis of light chains and anti-light chains*



electrophoresis of sulfonated heavy chains showed only one slow moving band corresponding to the heavy chains, indicating that the separation of heavy and light chains was almost complete (fig. 4). Sulfonated myosin showed one slow

moving band (heavy chains) and two fast moving bands (light chains) in electrophoresis. The light chains prepared from the myosin were separated usually into two bands in SDS-gel electrophoresis. These molecular weights were 27,000 (L-1) and 17,000 (L-2). However, in addition to these two bands, one having a molecular weight of 14,000 (L-3) sometimes appeared. When L-3 appeared in electrophoresis, the density of the band corresponding to L-1 was somewhat reduced.

In immunodiffusion with anti-light chains, the light chains produced two lines (fig. 5). At a high concentration of antigen (0.4 mg/ml light chains in fig. 5), the two lines were fused and one diffuse line was appeared around the antibody well. Anti-light chains also reacted with myosin and produced one line corresponding to light chains. The line corresponding to heavy chains was not observed. This fact indicated immunologically that the separation of heavy and light chains was almost complete.

Two peaks were also observed in quantitative precipitin analysis (fig. 6). These results indicated that two kinds of light chains were observed by immunological analyses.

Discussions

The results of the experiments on the purity of myosin showed that a highly purified myosin was obtained by the purification procedures described in this paper. The further applications of the ionic exchanger chromatographies after the procedures based on the solubility of myosin, such as precipitation at low ionic strength, fractionation with $(NH_4)_2SO_4$ and ultracentrifugation, resulted in a highly purified myosin preparation.

The myosin produced one peak in quantitative precipitin analysis with the homologous antibodies. Our result was different from that of HORVÁTH & SHAFIQ, who observed two peaks; one corresponding to light chain-free myosin and the other corresponding to light chains. We observed one peak with a broad shoulder in the analysis with the partially purified myosin before chromatography on DEAE-Sephadex A-50 and the homologous antibodies, and the shoulder was completely disappeared by the chromatography (fig. 3). From these results, the difference between our result and that of HORVÁTH & SHAFIQ is due to the purity of the myosin preparations.

As to the production of two precipitin lines by myosin and anti-myosin in immunodiffusion (fig. 2), LOWEY & STEINER explained in terms of the dissociation of some light chain components by anti-light chains. On the other hand, HORVÁTH & GAETJENS explained this phenomenon in terms of the presence of free light chain components not bound to myosin in myosin preparation. On

the basis of these views, it is expected that two peaks are observed in quantitative precipitin analysis as well as two lines in immunodiffusion. However, the highly purified myosin produced only one peak in the analysis as described above. Therefore, it is suggested that the difference between our results of the immunological experiments is caused by dissociation of light chain components from myosin only in agar gel. It is not known what kind of factors in agar promote the dissociation of light chain components from myosin. The effects of ionizing groups in agar seem to be excluded by the fact that immunodiffusion using agarose instead of agar gave the same result.

L-3 sometimes appeared with an accompanying decrease in the density of L-1 in the electrophoresis of light chains. Recently HORVÁTH & GAETJENS have reported that L-1 and L-3 were immunologically identical. This fact and the observations in this paper implied that L-3 was a breakdown product of L-1. L-3 may be produced from L-1 during the preparation of light chains. Many investigators have reported that the light chains consisted of three components (L-1, L-2 and L-3). The difference from our results may be explained in terms that the guanidine-HCl treatment of myosin to prepare the light chains reduces the breakdown of L-1 compared to the alkali treatment.

LOWEY & STEINER have reported one diffuse precipitin line in immunodiffusion with light chains and the homologous antibodies. However, HORVÁTH & SHAFIQ observed two precipitin lines. We observed two lines under suitable conditions, especially a protein concentration of antigen.

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