# MOLECULAR SIEVE CHROMATOGRAPHY OF MYOSIN ON AGAROSE COLUMNS

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SUMMARY Myosin monomer can be separated from aggregated myosin and heterogeneous low molecular weight contaminants by chromatography on 4% or 2% agarose. The monomer was characterized by its ATPase activity, which was constant across the peak, and its molecular weight (about 465,000). Chromatographic studies of aged samples show that aggregation is accompanied by the release of low molecular weight material. Molecular sieve chromatography provides a new approach to the purification of myosin which should be useful in studies on its molecular structure.

## INTRODUCTION

Molecular weight homogeneity of starting material is especially relevant for structural studies on the muscle protein myosin for in solution this protein irreversibly polymerizes to high molecular weight aggregates (1-3). Furthermore, there is currently a great deal of interest in the low molecular weight components that appear to be a part of the myosin molecule critical to its structure and function (4-10). To evaluate the role of this latter material, it is necessary to insure the removal of contaminants that may be confused with these low molecular weight components (10). For these reasons we have explored the use of molecular sieve chromatography as an adjunct to precipitation methods and ion exchange chromatography (11). Use of the method has permitted us to follow the aggregation process and draw some preliminary conclusions concerning its nature.

## EXPERIMENTAL

*Preparation of Myosin.* Myosin was prepared from the psoas muscle of young rabbits as described by Kielley and Bradley (12) and Kielley and Harrington (13)

and included the 40% $(NH_4)_2SO_4$ precipitation to remove actomyosin. All solutions were made with glass distilled water and all procedures were carried out at 4°C. The final solution, containing about 1% myosin in 0.5 M KCl, 0.01 M Tris-HCl, pH 7.5 (TK buffer), was clarified by centrifugation at 100,000 g for 2 hours. Concentrations were determined from the absorbance at 280 mμ using an extinction coeffient of 5.43 (14) for a 1% solution in a 1-cm cell. The absorption spectrum showed a maximum at 278 mμ and the 280/260 mμ ratio was 1.71 suggesting minimal contamination with nucleic acid.

Molecular Sieve Chromatography. Columns 15 mm × 120 cm with capillary tips (15) were packed with 4% (Bio-Gel A15) or 2% (Bio-Gel A50) agarose over a short bed of fine glass beads supported on a glass wool plug. The columns were poured in one section with a slurry of agarose in TK buffer at a rate that did not exceed the final flow rate of the column, 12-15 ml/hr. When the packed column height was about 110 cm, the column was allowed to flow under gravity overnight with a hydrostatic head equal to the height of the column. The flow rate was then measured to ensure that it was greater than 15 ml/hour and the rate was metered at 12-15 ml/hour at the bottom of the column with a Buchler peristaltic pump. The effluent was led through a micro flow cell with a 1-cm light path in a Beckman DB-G spectrophotometer and then to a fraction collector. Connections were made with Tygon tubing, 0.05 inch bore. Absorbance at 278 mμ or, for greater sensitivity, at 220 mμ was recorded continuously on a Beckman 10" chart recorder. The entire assembly except for the recorder was kept in a cold box (Gilson) at 4°C. The sample was unually applied in a volume of 1-2 ml and included a small amount of $^3H_2O$ as a measure of the included volume of the column.

ATPase activity. Enzyme activity was assayed directly in the fractions collected from the molecular sieve column after the addition of EDTA (adjusted to pH 7.5 with KOH) and ATP (Sigma) to give a 10 mM final concentration of each (12). Samples were incubated at 37°C for 5 min and the reaction was stopped by the addition of 2 ml of a solution containing 1.5 ml 3N $H_2SO_4$ and 0.5 ml 2.5%

ammonium molybdate. The liberated orthophosphate was measured from the absorbance at 820 mμ after the addition of 0.5 ml 10% ascorbic acid and incubation at room temperature for 1 hour (16).

Sulfhydryl Group Assay. The -SH content was estimated by the general method of Ellman (17) using a 50-fold excess of DTNB [5,5'-dithiobis-(2-nitrobenzoic acid)] (Aldrich), 1 mM EDTA and 5 M guanidine· HCl. After 30 min at room temperature the reaction was complete and the absorbance was measured at 412 mμ. The number of -SH groups was calculated using a molar extinction coefficient of 13,600 (17).

Molecular Weight Determination. The molecular weight of myosin in fractions from the molecular sieve column was determined in the Spinco Model E ultracentrifuge by the meniscus depletion method of Yphantis (18). Column heights were about 3.0 mm in a six-hole Yphantis cell placed in an An-D titanium rotor. Concentrations of 0.01-0.05% were employed. FC-43 was not used. The samples were initially overspeeded for 6-8 hours at 12000 rpm. Equilibrium was reached after 2 days at 8000 rpm and 1°C. Measurements of the Rayleigh fringes and calculations were those of Yphantis (18) as previously described (19). A value of 0.720 for the apparent partial specific volume was used (20).

## RESULTS

Chromatography of a freshly prepared myosin sample on 2% (Fig. 1) or 4% agarose (Fig. 2) produces a major peak with constant specific ATPase activity across its center, a relatively small peak at the excluded volume with markedly less ATPase activity, and heterogeneous material of apparent low molecular weight appearing in the effluent between the major peak and the included volume of the column. The major peak, which can be attributed to monomeric myosin, is better separated from the higher molecular weight material on the 2% than on the 4% agarose. The resolution was best when small amounts of myosin, about 2 mg, were chromatographed. However, resolution was satisfactory for the preparation of monomer from as much as 50 mg (in a 5-ml volume) of sample.

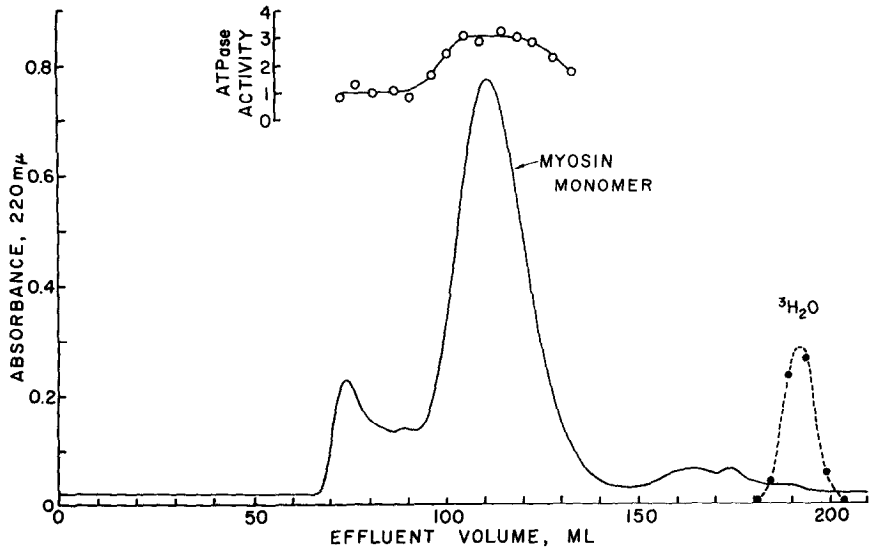


Fig. 1. Chromatography of a 2-day old myosin preparation on a 1.5 x 110 cm column of 2% agarose. Approximately 2 mg of protein was applied to the column. The column was eluted at 14 ml/hr and absorbance was measured continuously in the effluent. ATPase specific activity (o, $\mu g$ P/5 min/$A_{220}$) was measured in the effluent fractions. The sample contained $^3H_2O$ (dash line, arbitrary units as a measure of the included volume of the column.

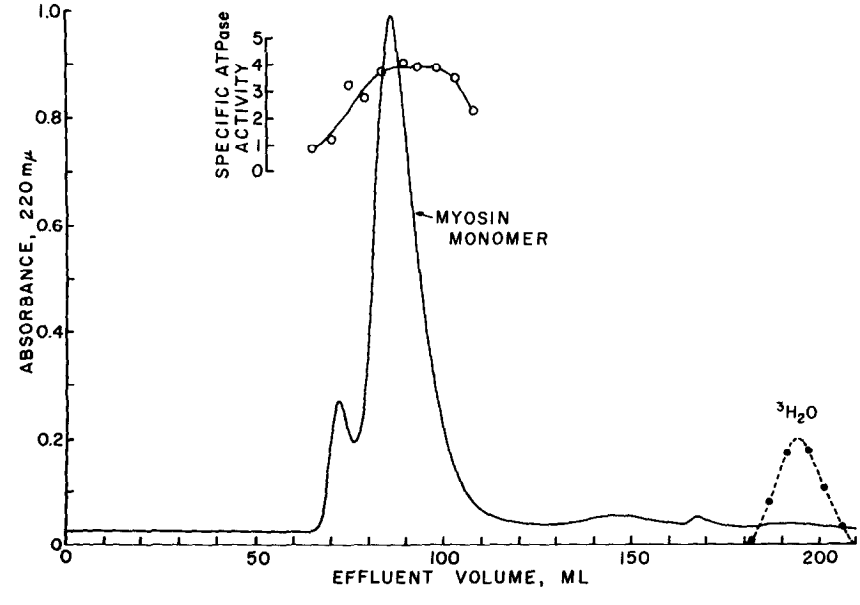


Fig. 2. Chromatography of a 2-day-old myosin preparation on a 1.5 x 110 cm column of 4% agarose under the same conditions as in Fig. 1.

The sample used to obtain the chromatograms in Figs. 1 and 2 was 2 days old counting from the time the animal was sacrificed. Samples kept at 5°C for longer periods were examined by chromatography on 4% agarose. The areas attributable to monomeric myosin, aggregated myosin and low molecular weight material were determined by planimetry (Fig. 3). The amounts of aggregate increased from an extrapolated value of 5% at zero time to about 30% in 40 days. The amount of low molecular weight material also increased at nearly the same rate, from about 4% to 20% in 40 days. Since the extinction coefficients of these components are not known, the area measurements are only roughly indicative of absolute amounts. At all times the low molecular weight material appeared heterogeneous in that it did not form a single well defined peak. The rate of polymerization was found to be dependent on the concentration of myosin. Dilution from about 10 mg/ml to 1 mg/ml reduced the rate of appearance of aggregate as measured by chromatography by about one-half. Monomer purified by

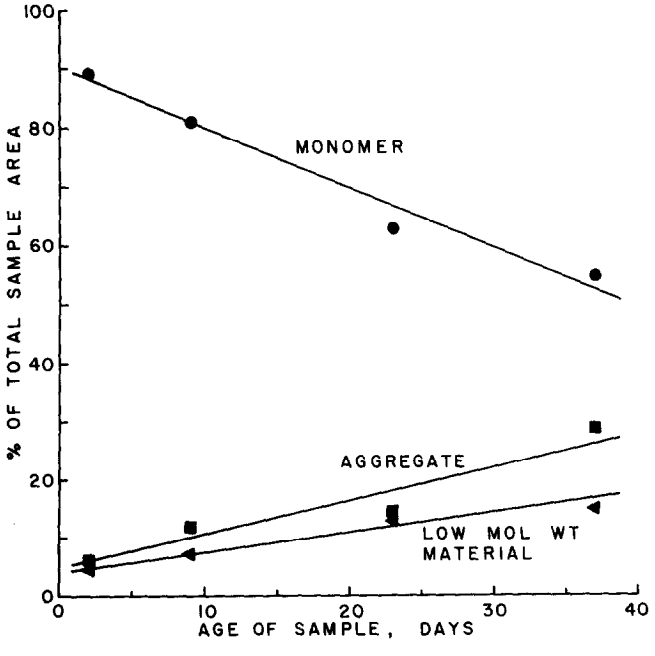


Fig. 3. Relative amounts of myosin monomer (●), aggregate (■) and low molecular weight material (◀) as a function of age of the sample. The amounts were determined by area measurements of chromatograms (see Fig. 1) monitored at 280 mμ.

TABLE I

Molecular Weight of Myosin after Molecular Sieve Chromatography

| | Molecular Weight ($M_w$) | | |
|---|---|---|---|
| Conc, % | Whole Cell | At c=o | At c=b ($M_z$) |
| 0.015 | 462,000 | 440,000 | 487,000 |
| 0.03 | 463,000 | 412,000 | 544,000 |
| 0.04 | 470,000 | 443,000 | 505,000 |
| Average | 465,000 | 432,000 | 512,000 |

chromatography and allowed to stand, on rechromatography showed the production of aggregate and low molecular weight material in much the same manner as the original myosin.

Examination of the protein in the major peak by high speed sedimentation equilibrium showed it to have a weight average molecular weight of about 465,000 which was apparently independent of concentration (Table I). This value is similar to values obtained for myosin in more extensive studies (see, for example, ref. 21). The molecular weights extrapolated to zero concentration within the cell (c=o) and to the bottom of the cell (c=b) indicate some heterogeneity of the sample which presumably developed during the 2 days required to reach equilibrium in the ultracentrifuge. The age of the sample prior to chromatography had no significant effect on the molecular weight of the myosin monomer obtained in the major peak. Since the chromatographic studies indicate that both high and low molecular weight material formed with time, the molecular weight averaged over the whole cell is probably closest to the correct value.

The sulfhydryl content of monomeric myosin after chromatography was 7.5 -SH groups/$10^5$g in good agreement with published values (13,22). The high molecular weight aggregate had a lower content, 4.7 -SH groups/$10^5$g.

## DISCUSSION

Molecular sieve chromatography provides a new method for separating monomeric myosin from aggregated myosin as well as from at least a part of associated low molecular weight material. The continued formation of low molecular weight

material on standing suggests that its formation is associated with the aggregation process. In the absence of more complete data, it is not possible to state whether the material we see is a structural component of myosin (4-10) released by partial denaturation or is material formed by nonspecific degradation. Both processes may be involved. Therefore, it is evident from our results as well as other studies (10) that it is necessary to distinguish carefully between "light chains" that may be a structural part of the molecule and low molecular weight contaminants or degradation products. Molecular sieve chromatography of native myosin provides a technique for the removal of the latter type of material.

Recent studies (21) suggest that myosin in solution under usual conditions exists in a rapid monomer-dimer equilibrium. Such an equilibrium would not have been detected by our chromatographic studies. The measured molecular weight is also not inconsistent with a monomer-dimer equilibrium. In the concentration range employed the effects of a monomer-dimer equilibrium and nonideality would be opposite and approximately equal. An apparent independence of molecular weight on concentration and an extrapolated value of about 480,000 would be predicted from the data of Godfrey and Harrington (21). This is within experimental error of the observed value of 465,000. Thus, the good agreement between our value and the extrapolated value of 468,000 (corrected for nonideality and monomer-dimer equilibrium) found by Godfrey and Harrington (21) is in part fortuitous.

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