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# APPLICATION OF HYDROXYAPATITE CHROMATOGRAPHY TO ALKALINE MYOSIN SOLUTIONS*,**

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## SUMMARY

The light components present in alkali-dissociated myosin were separable from the major fraction by alkaline hydroxyapatite chromatography at pH 10.5 with a minimum yield of 9%. On alkaline hydroxyapatite columns in the pH range of 10–12.5, as the pH was increased, the large myosin fraction eluted at progressively lower $P_i$ concentrations in accord with the behavior of proteins in general on hydroxyapatite.

Chromatography on pH 6.8 columns separated alkali-dissociated and then neutralized myosin into two fractions. The first contained "free" light components. The second contained the major fraction (generally believed to consist of two identical heavy subunits) combined with light components. When this second fraction was split into three parts and individually rechromatographed, each sample eluted with a fairly narrow and symmetrical peak. The sample from the peak part exhibited decreased binding to the hydroxyapatite; that in the trailing edge eluted at a similar $P_i$ molarity as did native myosin. Polyacrylamide electrophoresis indicated that there were more light components eluted with the heavy subunits in the material in the trailing edge than that in the peak.

## INTRODUCTION

Above pH 10.5 two or more low-molecular-weight components ("light chains") are dissociated from myosin, leaving a "heavy" double-chain core[1–4]. Separation of

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the light and heavy components has been accomplished by precipitation of the latter by dilution with water[1,2] or by addition of potassium citrate[3]. With both procedures the precipitated heavy component is contaminated with varying amounts of coprecipitated or entrapped light components. A complete separation of the light from the heavy subunits would be desirable for studying the structure and function of the myosin subunits.

This study was initiated for the purpose of determining whether hydroxyapatite (HAP) chromatography[5-8] might have application for this purpose. It was found that the separation could be achieved and in addition that alkaline treatment of myosin resulted in changes which could be detected by the behavior of the myosin components on the HAP column.

## MATERIALS AND METHODS

Deionized glass-distilled water was used routinely. HAP was prepared by the method of RICHARDS AND BERNARDI (unpublished results), which is a modification of those of TISELIUS *et al.*[5] and ANACKER AND STOY[9]. DEAE-Sephadex A-50 was purchased from Pharmacia. Tris, ATP, and histidine-HCl were obtained from Sigma, and DTT from Calbiochem. Other chemicals used were reagent grade.

In all experiments myosin samples were at a temperature of 0–4°.

Myosin samples were prepared from rabbit (New Zealand white) back muscle by the extraction method described by TONOMURA *et al.*[10], followed by purification chromatography on DEAE-Sephadex A-50 columns[11].

HAP columns were packed as described by BERNARDI[12].

Further chromatography on neutral HAP columns was performed with the purified myosin in 0.2 *M* $P_i$ or 0.5 *M* KCl–0.01 *M* $P_i$ buffer solutions at pH 6.8. Protein was eluted with a linear $P_i$ gradient. The myosin fraction was precipitated by dialysis against 0.05 *M* KCl–$P_i$ buffer, and the resulting precipitate was dialyzed into the desired solvent.

Alkali-dissociated myosin was prepared for separation on neutral HAP columns by dialysis of purified myosin samples against pH 11 buffers for 9–36 h. The pH of the samples was then adjusted to 6.8 by dialysis for 24 h against the buffer to be used for chromatography on neutral HAP columns.

For chromatography on alkaline HAP columns both the columns and samples were prepared at pH values which ranged from 10 to 12.5. The purified myosin samples were adjusted to the desired pH by the careful addition of 1 *N* KOH. When 0.001 *M* $P_i$ was used in the starting buffer, the pH levels of the eluted solutions varied. The desired pH was achieved by using a higher molarity in the rinsing solvent or by the addition of glycine to the eluting buffer.

Volumetric dilutions of protein solutions were routinely scanned from 350 to 245 nm in 1-cm quartz cells on a Cary 15 automatic recording spectrophotometer. The absorbance at the peak ($A_{279\ nm}$) was corrected for light scattering material by linear extrapolation from 350 to 320 nm. The absorbance of column fractions at 280 nm was measured with a Zeiss M4 QIII spectrophotometer.

ATPase activity was measured by the procedure used by RICHARDS *et al.*[11]. To determine if reconstituted myosin had ATPase activity, 3.0 ml of 0.16% light com-

ponents was added to 3.5 ml of 0.8% heavy components. The pH of the mixture was raised from neutrality to pH 10.7 with 1 *N* KOH, stirred for 4 min and then dialyzed back to pH 7.5.

Polyacrylamide gel electrophoresis analyses were performed on 2.9% gels in glycine–Tris buffers containing 0.3% sodium dodecyl sulfate, pH 8 (PATERSON AND STROHMAN[13]). Approx. 1 mg/ml protein for myosin or the heavy component fraction, and 0.1 mg/ml protein for the light chains were dialyzed against 0.3 *M* NaCl, 0.001 *M* $P_i$ prior to electrophoresis.

Sedimentation velocity experiments were performed with a Beckman Spinco Model E at 59,000 r.p.m., and patterns were observed with schlieren optics at an 80° phase–plate angle.

## RESULTS

### *HAP chromatography at pH 6.8*

A typical elution pattern obtained when myosin, which had been purified by DEAE-Sephadex chromatography, was eluted from HAP at neutral pH is shown in Fig. 1. The same elution profile was obtained when the solvent was $P_i$ or $P_i$–KCl.

Two typical HAP chromatograms of myosin solutions which had first been adjusted to alkaline conditions and then brought to pH 6.8 are shown in Figs. 2a and b. The first peak in Fig. 2a lacked a protein-type absorption spectrum and was not investigated further. The second peak in Fig. 2a and the small peak in Fig. 2b contained about half of the light components and no heavy components as determined by

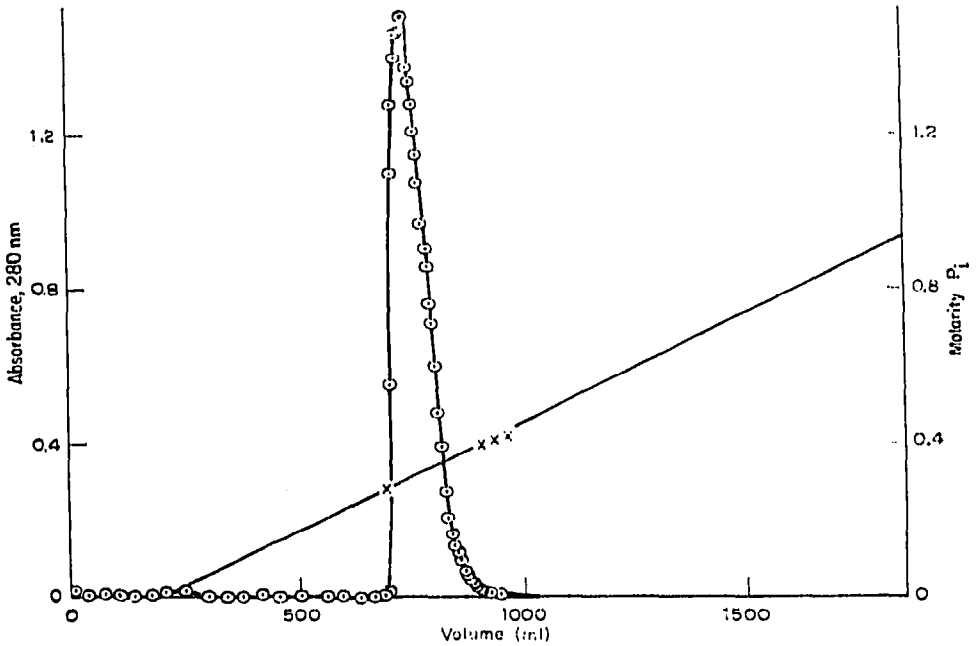


Fig. 1. HAP column chromatogram of rabbit myosin. Column 2.5 × 40 cm; myosin applied, 328 mg in 200 ml; elution system, gradient $P_i$ buffer (400 ml 0.2 *M* $P_i$ plus 400 ml 0.5 *M* $P_i$), pH 6.8. —⊙—, Absorbance; — × —, $P_i$ molarity.

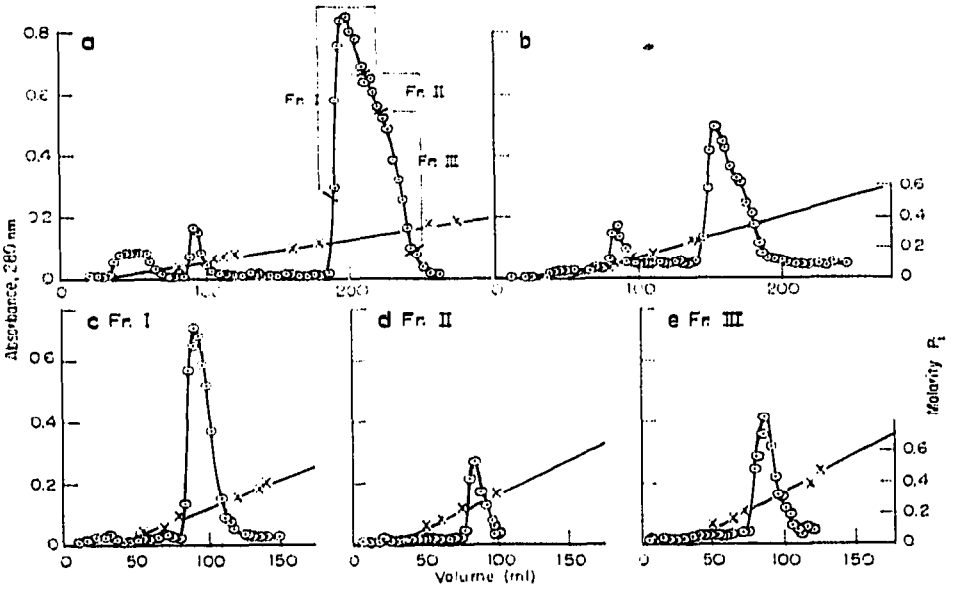


Fig. 2. HAP column chromatograms of alkali-dissociated myosin at pH 6.8. (a, b) Fractionation after dialysis back to neutrality. (a) Column, 2.5 × 40 cm; protein applied, 75 mg in 29 ml; elution system, KCl–gradient $P_i$ buffer (400 ml 0.5 *M* KCl–0.01 *M* $P_i$ plus 400 ml 0.5 *M* KCl–0.6 *M* $P_i$), pH 6.8; (b) Column, 1.5 × 10 cm; protein applied, 21 mg in 13 ml; elution system, KCl–gradient $P_i$ buffer (125 ml 0.5 *M* KCl–0.001 *M* $P_i$ plus 125 ml 0.5 *M* KCl–0.6 *M* $P_i$), pH 6.8. (c, d, e) Rechromatography of fractions I, II, and III from a. Columns, 1 × 23 cm; elution system, KCl–gradient $P_i$ buffer (100 ml 0.5 *M* KCl–0.01 *M* $P_i$ plus 100 ml 0.5 *M* KCl–0.6 *M* $P_i$), pH 6.8. (c) Fraction I applied, 30 mg in 20 ml. (d) Fraction II applied, 8 mg in 8 ml. (e) Fraction III applied, 12 mg in 16 ml. —○—, Absorbance; —×—, $P_i$ molarity.

polyacrylamide gel electrophoresis. Sedimentation velocity analysis of a separate myosin sample similarly treated showed that about half of the light chains had reassociated with the heavy components. The amount of recombination of light with heavy components after dialysis to neutrality was not constant but depended upon the time of exposure to the alkaline pH[2,14].

The large fraction began to emerge at about 0.24 *M* $P_i$ and developed a broad and skewed peak (Figs. 2a and b). This peak was separated into three fractions (Fr. I, Fr. II, and Fr. III in Fig. 2a), which upon rechromatography, eluted with rather narrow symmetrical peaks and at about the same $P_i$ concentrations as they did in the original chromatogram (0.23, 0.26, and 0.3 *M* $P_i$, respectively, see Figs. 2c, d and e). The elution molarity of Fr. III was similar to that of native myosin (0.3 *M*). Polyacrylamide gel patterns revealed that Fr. III contained bands on the gels that were at similar positions as bands of native myosin with respect to both small and large subunits while Fr. I contained mainly bands that were indicative of the large subunits.

*Behavior of myosin on alkaline HAP columns*

As the pH values of the HAP columns were raised over 10.0, there was an increase in the amount of UV absorbing material in the void volume (Fig. 3). At pH 10.5, 9 ± 2% of light components appeared in the void volume (average of three HAP runs, *cf.* Fig. 3b). The light components were distinctly separated from the heavy

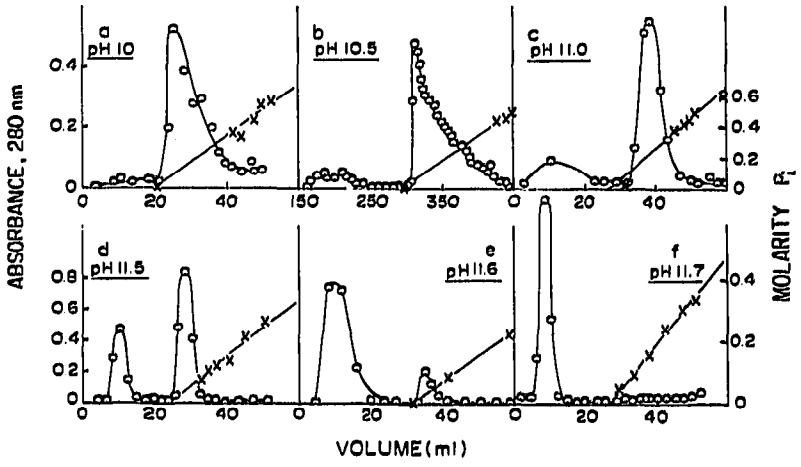


Fig. 3. HAP column chromatograms of alkali-treated myosin at pH 10–11.7. pH is shown in Figs. a–f. Columns, 1 × 10 cm for a, c, d, and f; 2.5 × 40 cm for b, and 1.5 × 30 cm for e. Total protein applied: a, 12 mg in 2 ml; b, 50 mg in 10 ml; c, 10 mg in 2 ml; d, 13 mg in 2 ml; e, 16 mg in 2.7 ml; f, 12 mg in 2 ml. Elution systems, 0.5 *M* KCl–gradient $P_i$ buffer, as follows: a, c, d, 40 ml 0.001 *M* $P_i$ plus 40 ml 0.8 *M* $P_i$; b, e, 200 ml 0.01 *M* $P_i$ plus 200 ml 0.8 *M* Pi; f, 40 ml 0.01 *M* $P_i$ plus 40 ml 0.8 *M* $P_i$. The total protein that eluted before gradient was applied amounted to: a, none; b, 9%; c, 15%; d, 32%; e, 88%; f, 100%.

fraction which emerged only after the salt gradient was applied. Polyacrylamide gel patterns confirmed the identities of the light and heavy components. On the alkaline HAP columns the major fraction emerged at lower $P_i$ molarities than on the pH 6.8 columns indicating that the affinity of myosin for HAP had been reduced, even at pH 10 where the light components had not yet been released. This decrease in affinity for HAP with increasing pH is a characteristic of proteins in general. At higher pH levels there was a gradual sharpening of the peak of the large fraction, along with a decrease in the $P_i$ molarity necessary to elute it. Above pH 11 the large fraction became increasingly more evident in the void volume along with the small and at pH 11.7 and above, all fractions eluted in the void volume.

*ATPase analysis*

Myosin samples incubated at pH 11.0 and dialyzed back to pH 7 had no significant ATPase activity. The separated large and small fractions from a myosin sample run on HAP at pH 10.5 showed no appreciable enzymatic activity individually or when combined, in agreement with the results of DREIZEN AND GERSHMAN[15]. However, the recombination of myosin fractions from LiCl–citrate dissociation is reported to result in substantial recovery of the original ATPase activity[15–18].

*Polyacrylamide gel analysis*

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate was used to establish the presence of light and/or heavy subunits in the fractions from HAP columns. Fig. 4c-3 is a photograph of rabbit myosin which had been chromatographed on DEAE-Sephadex A-50 columns. Fig. 4c-4 is a pattern of a chicken myosin sample prepared by PATERSON. Both show slow bands that represent the heavy subunits and


Fig. 4. Disc gel electrophoresis of components in alkali-dissociated myosin after separation on HAP columns. In a, b, c, the fractions applied were: (a) -1, -2, 100 ng each from void volume of Fig. 3c; -3, -4, from $P_i$ gradient of Fig. 3c, 25, 50 ng, respectively; (b) -1, -2, recombined fractions a-1 and a-3, 50 ng, 100 ng, respectively; -3, -4, fraction from void volume of Fig. 3f, 50 ng each; (c) -1, same as b-3 and b-4, 50 ng each; -2, fraction from $P_i$ gradient fraction of Fig. 3f, 100 ng; -3, rabbit myosin, 15 ng; -4, chicken myosin, 15 ng.

two fast bands which are indicative of light chains[13]. The gel results with alkali-treated myosins showed that the pH at which a separation between light and heavy components can be made on HAP columns was about pH 10.5. Photographs of typical void volume fractions containing mainly light chains are shown in Figs. 4a-1,

b-3, and c-1. Whether two or four bands were visualized (Figs 4a-1, and b-3, c-1, respectively) probably depended on the amount of sample that was applied to the gel. Even purified myosin appeared to contain only two fast bands when 15 $\mu$g was applied. As an example of underloading of gels, no fast band was detected on the photograph of the $P_i$ gradient fraction of a pH 10.5 HAP run (which normally contained the heavy fraction only) when 25 $\mu$g was applied (Fig. 4a-3) but a fast band was observed on the photograph of the same sample when 50 $\mu$g was applied (Fig. 4a-4). Even the $P_i$ gradient fraction of a pH 11.7 column run showed some small component when 100 $\mu$g was applied (Fig. 4c-2).

## DISCUSSION

Light chains of 9 ± 2% in rabbit myosin can be separated from the heavy components on HAP columns at pH 10.5. This yield compares roughly with those reported by several other authors[2,14,19,20]. Polyacrylamide gel electrophoresis showed that the light chains emerged in the void volume of the HAP chromatogram; the heavy components did not appear until after application of the salt gradient.

The large components of myosin are presumed to be made up of two identical subunits, molecular weight 194,000–220,000 (refs. 1, 2, 19). The similarity of sedimentation coefficients at pH 7 and pH 12, indicates that exposure to pH 12 does not cause separation of these two chains. Similar results have been reported by others[1,14]. TONOMURA *et al.*[21] found a decrease in myosin helix content from 60 to 8% in high concentrations of LiBr at neutral pH. Some preliminary evidence[4] indicates that 4 *M* LiBr acts differently on myosin at pH 11 than at pH 8.

Our data suggest that a combination of high salt and high pH may hasten a denaturation process of myosin. HAP appears to distinguish between extents of denaturation. At the higher pH levels of protein samples and columns, the large components elute at increasingly lower $P_i$ molarities in accord with the behavior of proteins in general on HAP. Sedimentation studies have shown that the myosin molecules exhibit an increased concentration dependence and increased aggregation tendency as the pH is raised[1]. Thus the decrease in HAP binding with increase in pH is possibly the result of these known configurational changes leading to a loss of available carboxyl group binding sites. BERNARDI AND KAWASAKI[8] had suggested that a gradual decrease in the binding of collagen to HAP columns at increasingly higher temperatures could be explained by such a mechanism.

Evidence was obtained that myosin can be separated into native-like and partially denatured molecules. Myosin, raised to pH 11 and then returned to pH 6.8, eluted from a pH 6.8 HAP column with a broad skewed major fraction. This fraction was split into three parts so that the protein solutions from the trailing edge, the peak, and the cut half-way in between could be individually rechromatographed. Each sample emerged at a $P_i$ molarity similar to its position in the original chromatogram with a sharp, symmetrical peak (see Figs. 2a, c, d, e). The material at the trailing edge acted like the original myosin since (1) it eluted at a similar $P_i$ molarity, about 0.3 *M*; and (2) polyacrylamide electrophoresis showed that it contained more light chains than did the material in the peak. It may be inferred that the different $P_i$ elution molarities exhibited by the trailing edge, the peak, and the middle fraction represent differences in their secondary structure.

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