A NEW METHOD FOR PRODUCING MYOSIN SUBFRAGMENT-1

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Summary: Myosin subfragment-1 (S1) was produced from myosin by digestion with papain while the myosin was "in situ" in a muscle fibril. S1 was extracted from the fibrils using Mg-PP_i, and chromatographed on Sephadex G-200. This S1 had sedimentation and diffusion coefficients similar to those found for S1 by previous workers. The Ca^{++} activated ATPase was 10-15 μmoles/gm-sec. The actin activated ATPase, extrapolated to infinite actin concentration, was 250 μmoles/gm-sec, and could be decreased by further papain digestion of the S1. Gel electrophoresis in the presence of sodium dodecyl sulfate showed one predominant heavy chain at 10^5 Daltons and three light chains at 22K, 18K and 16K Daltons.

Introduction: The myosin molecule consists of a long helical tail and two globular heads. The heads contain the enzymatic and actin-binding properties and thus have been the focus of a number of investigations. Early workers used trypsin to cleave off the two headed portion of the molecule, heavy meromyosin (HMM), and upon prolonged digestion to also produce single heads or myosin subfragment-1 (S1) (1). Kominz and coworkers (2) were the first to show that papain could be used more efficiently to cleave the S1's from the rest of the molecule. Subsequently, Lowey et al (3), using insolubilized papain, extensively studied the properties of S1. In addition to separating the head portion of the myosin from the tail, both trypsin and papain are capable of hydrolyzing many bonds, and questions remain as to whether the resulting subfragments contain the same properties as their corresponding moieties in the native myosin molecule (4,5).

In this paper a method for S1 production is described in which the papain digestion takes place while the myosin is "in situ" in the muscle fibril, with the heads attached to the thin filaments; thus protecting the actin-binding site. The heads, are subsequently released by Mg-PP_i affording a purification step. In addition to being far simpler and faster than previous methods, this

method produces S1 with high enzymatic activity in high yield.

Methods: Rabbit Psoas and back muscles were minced and suspended in 3-4 volumes of a 50:50 (v/v) mixture of glycerol:0.1M KCl, 0.01M Tris-maleate, pH 7.0. After one half hour in the glycerol mixture at 4°C, the muscles were either used or stored for up to two months at -20°C. Storage of the fibers did not appear to affect the properties of the S1. The glycerol-treated mince was diluted with 3-4 volumes of 0.1M KCl--0.01M Tris-maleate, pH 7.0 (this stock solution was used in all subsequent washes, etc.) and blended for 2 minutes in a Waring blender. The resulting fibrils were sedimented and resuspended three times in order to remove debris. The digestion of this suspension of fibrils (from 10-15 mg protein/ml) was carried out at 23°C using soluble papain (Worthington Co.). Unless otherwise stated, the digestion was done using 0.01 mg/ml papain for 10 minutes. The digestion was terminated by dilution with cold stock solution and the fibrils were again washed three times. The S1 was released from the fibrils with 1mM Mg-PP_i. S1 could also be released by ATP, but the ATP tended to solubilize other proteins in addition to S1 and did not appear to release more S1 than did PP_i. Following extraction, the fibrils were sedimented and the resulting supernatant was chromatographed on Sephadex G-200.

ATPase activity was measured in a pH-stat (Radiometer, Denmark) equipped with a 25°C water bath. Protein concentrations were determined by the Folin-Ciocalteu method (6).

Myosin was extracted by a modified Szent Györgyi method (7). For comparison with the present method, S1 was prepared from this myosin by the method of Lowey et al (3) using insoluble papain and insoluble myosin (papain:myosin = 1:2000 by weight, digestion for 10 min at 23°C) followed by chromatography on G-200. HMM was prepared by 10 minute digestion with trypsin (1:255, trypsin: myosin by weight), followed by precipitation of the LMM and chromatography on DEAE cellulose using a KCL gradient. Actin was prepared by the method of Cohen (8) from an acetone-dried muscle powder.

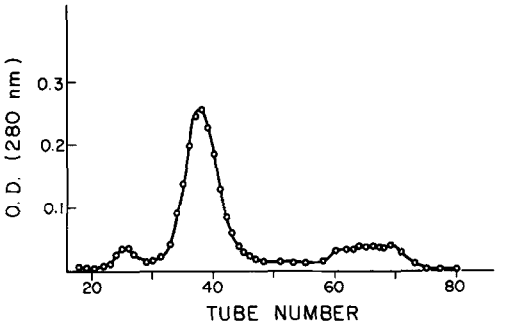

Fig. 1.

Chromatography of the crude S1 was carried out on Sephadex G-200, using an ascending flow of 15 ml/hr on a column 80 cm by 2.5 cm. The medium used was 0.15M KCl, 0.5mM $MgCl_2$, 0.5 mM PP_i, 10 mM Tris-maleate, pH 6.8.

Results and Discussion: The elution diagram for the chromatography of the crude S1 on Sephadex G-200 is shown in figure 1. Between 50-75% of the protein was eluted as a single peak at the same elution volume as that observed for S1 prepared by the method of Lowey et al (3). Mg-PP_i was included in the chromatography medium to dissociate the S1 from any actin fragments present. The crude S1 could also be chromatographed on a DEAE cellulose column using a KCl gradient to produce S1 with identical ATPase activities and patterns on gel electrophoresis as the S1 purified on Sephadex G-200. The work reported here, however, used S1 purified on the Sephadex column.

Sedimentation of the purified S1 produced a single schlieren peak in the ultracentrifuge, Fig. 2. The sedimentation coefficient was $s_{20,w}$ = 5.95 S at 1 mg/ml, in good agreement with that found by other workers (3, 9, 10, 11). The translational diffusion coefficient was $D_{20,w} = 4.3 \times 10^{-7}$ cm^2/sec at 1 mg/ml, a little larger than that found by other workers (9,11). Using the Svedberg equation an approximate molecular weight of 1.2×10^5 Daltons was calculated, in agreement with that found by previous workers (3).

Polyacrylamide gel electrophoresis with sodium dodecyl sulfate (SDS) on 5% acrylamide gels showed that the heavy chain of S1 can be cut into several fragments by the papain digestion. Short digestion times produced S1 which showed

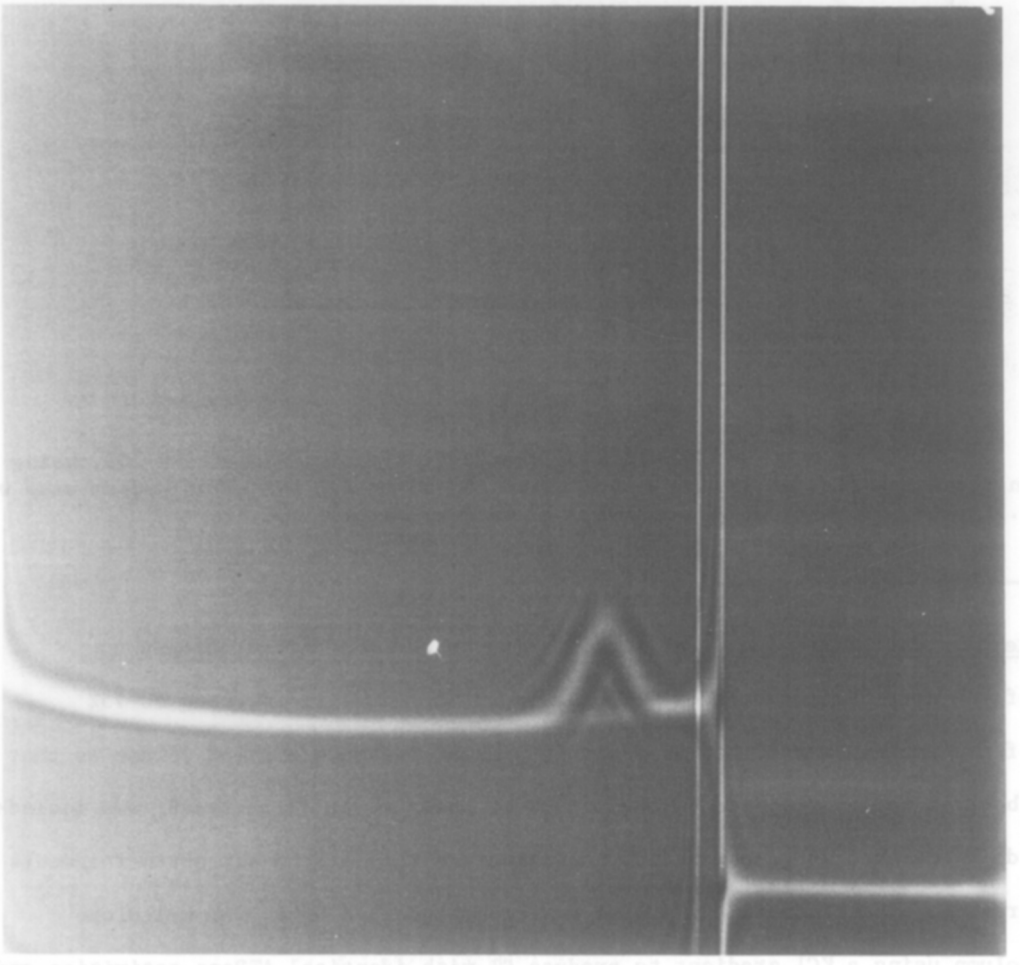

Fig. 2.

Sedimentation pattern for purified subfragment-1 at 59,780 RPM and 20°C. Sedimentation proceeds to the left. The medium was 0.15M KCl, 0.5mM $MgCl_2$, 0.5 mM PP_i, 10 mM Tris-maleate, pH 7.0; [S1] = 1 mg/ml.

predominantly one heavy chain of 10^5 Daltons, Fig. 3, No. 7. As the digestion time was increased, a second band at 7.5×10^4 Daltons became more pronounced, Fig. 3, No. 8-10. Densitometer scanning of the protein bound dye indicated that for short digestion times (i.e. Fig. 3, No. 1, 4, and 5) the 7.5×10^4 Dalton component represented only 4% of the total heavy chains. S1 produced by the in vitro digestion of myosin using insoluble papain (3) showed in addition several lighter fragments of the heavy chain, Fig. 3. No. 3. The fragments of the heavy chain pattern seen on SDS gels did not vary for samples

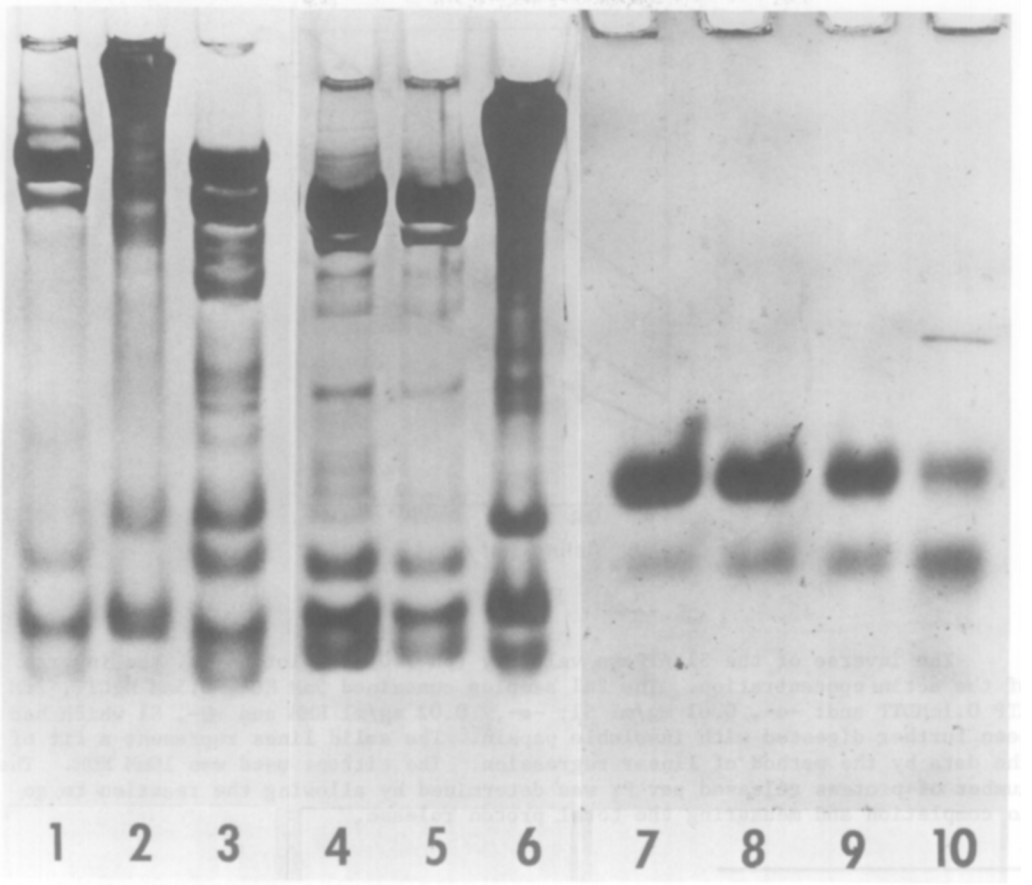

Fig. 3.

Polyacrylamide gel electrophoresis was done using slab gels. The Samples, 2-6 mg/ml, were incubated for 1 minute at 100°C in 1% SDS, 1% 2-mercaptoethanol, 2M urea and 0.3M NaCl. The electrophoresis medium contained 0.1% SDS and 0.43 M Tris-glycine buffer pH 8.8. Nos. 1-3 and 4-6 are both 10% acrylamide gels; 7-10 is a 5% acrylamide gel. The samples are: (1,4,5), S1 prepared from fibers using 0.01 mg/ml papain and a 10 minute digestion; (2,6), myosin; (3), S1 prepared from myosin; (7-10), S1 prepared from fibers using 0.03 mg/ml papain and 1.5, 5, 12 and 30 minute digestion times respectively-heavy chains only are seen in this gel.

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taken from the leading and trailing edges of the S1 peak in the Sephadex G-200 chromatography and all S1 samples migrated as a single peak in the ultracentrifuge. This is evidence that the heterogeneity of the S1 heavy chains represents cuts which do not fragment the S1 into smaller molecules; the fragments of the heavy chain remaining together, held by secondary interactions. This conclusion is supported by Stone and Perry (12) who have shown that S1,

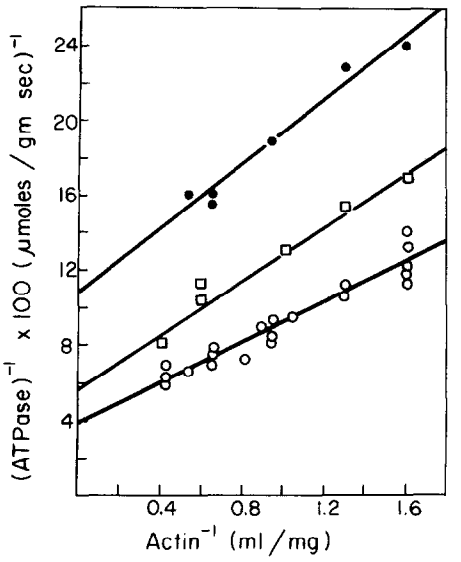

Fig. 4.

The inverse of the S1 ATPase velocity (pH 7.0) is plotted vs. the inverse of the actin concentration. The 2ml samples contained 5mM KCl, 0.5mM $MgCl_2$, 2mM ATP 0.1mMDTT and: -o-, 0.01 mg/ml S1; -●-, 0.02 mg/ml HMM and -■-, S1 which had been further digested with insoluble papain. The solid lines represent a fit of the data by the method of linear regression. The titrant used was 10mM KOH. The number of protons released per P_i was determined by allowing the reaction to go to completion and measuring the total proton release.

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which migrated as a single band in acrylamide electrophoresis without SDS migrated as several bands with SDS (12).

Myosin contains three light chains, as shown in Fig. 3 Nos. 2 and 6, whose molecular weights are taken as 16K, 18K and 25K (13). S1 produced from the fibrils also contained three light chains, however, samples No. 4 and 5 show that the heaviest of these S1 chains appears to have a molecular weight of 22K, i.e., less than the 25K chain observed in myosin. The more heavily digested S1 of Fig. 3, No. 3, shows four bands at 25K, 22K, 18K and 16K. More prolonged digestions of myosin in vitro produced a more pronounced band at 25K relative to the other light bands, and upon extensive digestion only the 25K band remained.

Interpretation of the light chain gel patterns can be confused by the presence of heavy chain fragments. Stone and Perry (12) have shown that papain digestion can produce a 26K S1 heavy chain fragment, in agreement with the pres-

ent study which finds that the density of the 25K chain depends on the extent of digestion. For the lowest degree of digestion (i.e. Figure 3. Nos. 1, 4 and 5) the 25K myosin light chain is absent. This could be a result of digestion of the 25K chain giving a 22K fragment. The possibility that this 25K chain has been lost in the purification can not however be eliminated.

The Ca^{++}-activated ATPase (0.6M KCl, 4mM Ca^{++}, 2mM ATP, pH 8.0) was 10-15 μmoles/gm-sec, which is lower than that found by previous workers (3) and is low compared to that expected from extrapolation from purified myosin preparations. No explanation of this discrepancy is apparent. However Ca^{++} activated ATPase can be both enhanced or killed by enzyme modification, making this assay a poor yard stick for enzymatic purity. The EDTA-ATPase was 90-110 μmoles/g-sec.

The ATPase of S1 in the presence of Mg^{2+} is activated many fold by F-actin. Eisenberg and Moos (14) were the first to show that a plot of $ATPase^{-1}$ vs $[actin]^{-1}$ gave a straight line for HMM. Figure 4 shows such a double reciprocal plot for HMM, S1 and S1 which has been further digested with insolubilized papain. The extrapolation to $[Actin]^{-1}=0$ gives $1/V_m$ where V_m is the maximum ATPase in the presence of saturating actin concentrations. The observed V_m for S1 as seen in Figure 4 is 250 μmoles/g-sec. This value is higher than that found by previous workers (3), with the exception of Onodera and Yagi whose S1 produced by digestion with chymotrypsin had a V_m of 238 μmoles/g-sec. S1 produced by the digestion of myosin had a V_m of 200 μmoles/g-sec and a gel pattern given by Figure 3. No. 3. To check whether degredation of S1 by papain can influence the actin activated ATPase, S1 was further digested using 0.01 mg/ml insolubilized papain for 10 min at 23°C. The heavy chain component of this S1 showed four bands of approximately equal intensity in gel electrophoresis, indicating slightly more digestion than that of sample No. 3 Fig. 3. As shown in Figure 4 the V_m of this S1 is lower than that before digestion. Thus the degradation of the S1 chains by papain can effect the actin activated ATPase. The V_m found for HMM varied from 90 to 110 μmoles/g-sec, in agreement with that found by other workers (14, 4). Per mole of myosin heads, this V_m is

low compared to the one found for S1. However, trypsin degrades the HMM heavy chain into several components as seen on gels, making it likely that the HMM V_m has been decreased from its true value for a native molecule.

The intercept on the abcissa of Figure 4 gives K^{-1}. The significance of K depends on the kinetic model chosen, and for one simple scheme has been interpreted as the dissociation constant for the actin S1-ATP complex (14). The data of figure 4 yield a value for K of 30 μmoles for S1 and 18 μmoles for HMM. The value of K for S1 has been decreased by further digestion by papain to 26 μmoles.

In summary, the digestion of myosin by papain can cut both the heavy and light chains into several fragments, affecting the actin activated ATPase. The present S1 preparation shows little degredation of the heavy chain and has a high actin activated ATPase.

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