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HETEROGENEITY OF SUBFRAGMENT-1 PREPARATIONS FROM MYOFIBRIL DIGESTION BY TRYPSIN*

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Summary : The preparation of subfragment HMM-S-1 of Rabbit skeletal myosin was achieved by a proteolytic digestion of myofibrils in the presence of EDTA. The product was purified on DEAE-cellulose and its characteristics were compared to those of HMM-S-1 prepared by other methods. In denaturing conditions the HMM-S-1 was shown to be fragmented into a small number of well defined polypeptides. The molecular weights of which were : 68000, 52000, 26000, 23500, 21000 and 14000 daltons. A certain degree of heterogeneity was revealed by the elution profile as well as the quantitative study of polyacrylamide gel electrophoresis.

Subfragment HMM-S-1 is usually obtained from myosin with either one of two techniques. a) A first hydrolysis with trypsin gives two fragments : light meromyosin (LMM) and heavy meromyosin (HMM). When HMM is subjected to a second tryptic digestion a new well defined subfragment HMM-S-1 can be obtained together with a certain amount of ill defined polypeptidic material (1). b) Papain yields HMM-S-1 directly and this globular part of myosin can be easily separated from the helical tail (2). A third method was introduced more recently (3) where myofibrils are digested with trypsin in the presence of EDTA. The insoluble complex HMM-S-1-actine is separated by centrifugation, then dissociated in the presence of pyrophosphate and Mg^{2+}. The soluble HMM-S-1 is collected in the supernatant after centrifugation.

A wealth of informations is available on HMM-S-1 obtained by either method a) or b) whereas the product of myofibril digestion needs to be further characterized. We report here a few observations made with techniques routinely used in this type of study.

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Abbreviations used : LMM : light meromyosin - HMM : heavy meromyosin
EDTA : ethylene-diamine-tetraacetic acid - DEAE : diethyl-amino-ethyl
SDS : sodium dodecylsulfate - DTT : dithiothreitol - DTNB : bis - (5-carboxy-4-nitrophenyl)disulphide.

MATERIAL AND METHODS.

Myofibrils from Rabbit psoas and back muscles were obtained according to Perry (4) in a final suspension of 10-15 mg ml^{-1} in a 0.1 M KCl, 0.02 M sodium borate buffer (pH 7.2). EDTA was added to a final concentration of 0.01 M, the pH was adjusted to 8.2 with 0.1 M NaOH, then DTT was added to a final concentration of 0.001 M. The suspension was incubated with trypsin (bovine pancreas, Worthington, 1 mg enzyme / 120 mg myofibrils) 1 hr at 24°C. The proteolytic digestion was stopped by adding a solution of trypsin inhibitor (soja bean, 2 mg inhibitor/ 1 mg trypsin). The reaction product was spun down for 2 hrs (100 000 x g) at 2-4°C, then resuspended in a 0.5 M KCl, 0.02 M BO_4^{2-}, 0.001 M DTT solution (pH 8.2) and sedimented again in the same conditions (from this point all operations were carried out in the cold room at 2-4°C). The sediment was dispersed with a Potter in a 0.5 M KCl, 0.005 M $MgCl_2$, 0.01 M pyrophosphate, 0.02 M borate, 0.001 M DTT solution (pH 8.2). The mixture was gently stirred overnight then centrifuged for 3 hrs (100 000 x g). An occasional layer of fat was eliminated and the supernatant (HMM-S-1 solution) was collected and dialyzed exhaustively against a 0.03 M Tris-HCl, 0.001 M DTT buffer (pH 7.7). A centrifugation (100 000 x g) was used to eliminate a light precipitate which may have appeared upon dialysis.

The HMM-S-1 was purified on a DEAE-cellulose column (Whatman DE 32 or DE 52, h : 500 mm; d : 19 mm). The column was equilibrated against the same buffer used for dialysis, then loaded with 120-200 mg proteins in 40-80 ml and washed with the equilibrium buffer. The proteins were eluted with a KCl gradient (0-0.15 M in the equilibrium buffer with a slope of 3×10^{-4} $M.ml^{-1}$ and a flow rate of 21-25 $ml.hr^{-1}$).

Polyacrylamide gel electrophoresis was performed according to Perrie and Perry (5) in 6.6 or 8 M urea. Polyacrylamide gel electrophoresis in the presence of SDS was carried out essentially as described by Weber and Osborn (6). ATPase activity was measured in a pH-stat thermostated at 25°C. All reactions were run in 0.5 M KCl, 0.01 M EDTA solutions.

RESULTS.

Elution profiles (fig.1) showed a marked dissymetry for which a confirmatory evidence was found in electrophoretic patterns in the presence of 8 M urea. However EDTA-ATPase activity was identical in the

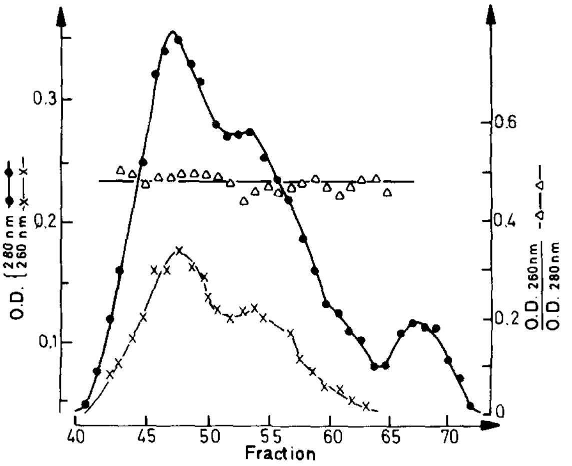


Figure 1. Chromatography of HMM-S-1 (preparation n° XV) on DEAE-cellulose. 50 ml of HMM-S-1 solution (1.25mg./ml). KCl gradient elution 0-0.15 M - 7 ml fractions, flow rate : 21-24 ml/hr.

first and last eluted fractions (3.2 ± 0.2 μmol Phosphate. min^{-1} mg^{-1}) an indication that the collected proteins were essentially HMM-S-1. Moreover a constant value for $E^{1\%}_{1\,cm}$ = 7.8 at 280 nm wavelength was obtained all along the elution profile. This value is comparable to that recorded by Yagi et al (7) ($E^{1\%}_{1\,cm}$ = 7.9) and is close among muscular proteins only to that of the subfragment S_n ($E^{1\%}_{1\,cm}$ = 8.00). At a pH of 7.7 the ratio of optical densities at 260 and 280 nm had a constant value of 0.48 ± 0.02 (fig.1) comparable to the value (0.6) given for HMM (8). Such a value exclude a contamination by nucleotides which could be suspected to distort the elution profile. An identical apparent molecular weight (124 000 ± 4 000) was found in the first part as well as in the last part of the peak. All these observations are good evidences that the eluted material was essentially HMM-S-1 along the elution profile.

Polyacrylamide gel electrophoresis in 6.6 M and 8 M urea.

In these dissociating conditions a clear evolution of the electrophoretic pattern along the elution profile was observed. An important fraction of the protein remains on top of the gel. Moreover two well resolved bands (U_A and U_B) could be observed. Their relative intensity varied significantly and regularly in the different fractions (fig.3). In each eluted fraction the amount of substance A and substance B respectively (expressed in arbitrary OD units) were computed from the percentage of U_A and U_B present in the gel. (Relative intensities were mea-

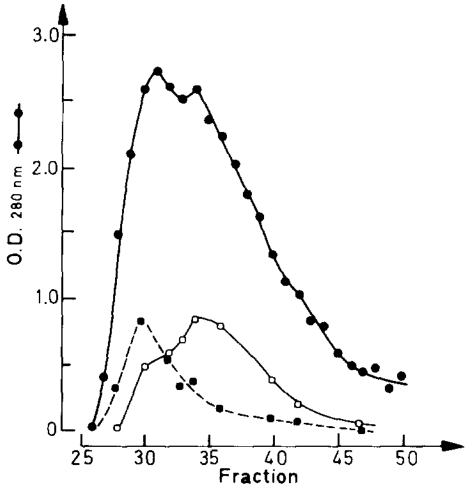


Figure 2. Chromatography of HMM-S-1 (preparation n° XIX) on DEAE cellulose. 80 ml of HMM-S-1 solution (4.8 mg/ml) - KCl gradient elution 0-0.15 M. 7 ml fractions : flow rate 21-24 ml/hr.

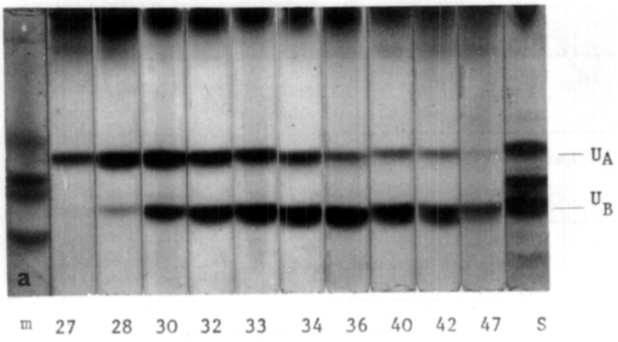
-O-O- OD of the effluent recorded at 280 nm;
-●-●- "reconstituted" elution profile for component A
□-□ "reconstituted" elution profile for component B (see text).

sured with a Vernon pH 1-5 densitometer). "Reconstituted" elution profiles for substances A and B are given in fig. 2. This profile seemed to coincide with the structure of the elution profile showing a correlation between the dissymmetry of the elution peak and the changing pattern of the electrophoretic picture along the elution profile.

Polyacrylamide gel electrophoresis in the presence of SDS.

Six bands were regularly observed. Their molecular weight were estimated by referring to a standard curve and the following average values were retained : H_1 : 68 000, H_2 : 52 000, L_1 : 26 000, L_2 : 23 500, L_3 : 21 000, B_3 : 14 000 daltons. H_1, H_2 and L_1 were always intense. A weak band with a molecular weight of about 90 000 daltons (HH) was found in some preparations and two light bands were occasionnaly visible at 16 000 and 18 000 daltons.

A quantitative study of the H_1/H_2 ratio showed important differences from one preparation to another (table 1). Along the elution profile H_1/H_2 decreased from a high value at the beginning of the elution to a lower value in the last fractions. A quantitative study of L_1/H_2 indicated that in spite of big differences for H_1/H_2 from preparations



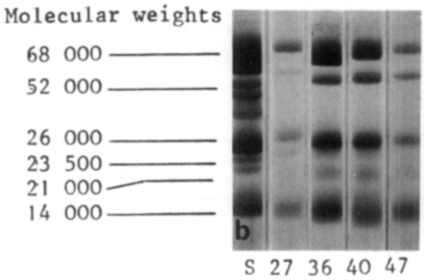


Figure 3a - Urea polyacrylamide gel electrophoresis. m : myosin S : crude HMM-S-1 before DEAE cellulose chromatography. 27-47 : fractions as shown in fig. 2

Figure 3b - SDS : polyacrylamide gel electrophoresis. S : crude HMM S-1 before DEAE cellulose chromatography. 27-47 : fraction as shown in fig. 2

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to preparations, L_1/H_2 assumed a nearly constant value close to 1. Moreover the ratio was also constant all along the elution profile.

DISCUSSION.

In similar conditions a dissymmetry of the elution peak from DEAE cellulose has been mentioned and a different electrophoretic pattern was obtained in the first fractions as compared to the last fractions (9). The present study brings a confirmation to these two observations and shows a satisfactory correlation between the structure of the elution profile and the "reconstituted" elution profile for components A and B obtained from electrophoresis. Another effect was recorded with the electrophoretic analysis of the chromatographic fractions in the presence of SDS. The first eluted fractions were significantly enriched in the 68 000 daltons component. Besides these evidences of a chromatographic heterogeneity no difference could be seen in the EDTA-ATPase activity and the apparent molecular weight in a non denaturing medium was identical within experimental error all along the elution profile. A measurement of the Mg^{2+} ATPase activity was not attempted as traces of actin were suspected in the last eluted fractions of some preparations.

Among the different bands observed in polyacrylamide gel electrophoresis in the presence of SDS it is reasonable to assume that two of them are light chains (the DTNB-light chain is supposedly excluded

TABLE 1.

Preparation	H_1/H_2		L_1/H_2	
	First fractions	Last fractions	First fractions	Last fractions
XIII	0.67	0.08	1.21	1.00
XIV	1.37	0.56	1.00	0.94
XIX	5.17	2.27	-	1.35
XIX'	4.61	3.00	1.07	1.21
XX	0.30	0.08	0.82	0.84

Quantitative electrophoretic study of components H_1, H_2 and L_1. Polyacrylamide gel electrophoresis in the presence of SDS. Proteins were stained with Coomassie Brilliant Blue and intensity recorded with a Vernon densitometer equipped with an integrator.

by DEAE cellulose chromatography). However a direct comparison of mobilities in parallel runs indicated that the HMM-S-1 alkali light chains might have a smaller molecular weight than the corresponding ones extracted from undigested myosin. On the other hand none of the heavy polypeptides corresponds to the normally expected heavy chain (90 000 - 100 000 daltons). It can be concluded that the proteolytic attack in the present experimental conditions results in a fragmentation of the heavy chain. Independently and in different experimental conditions two other studies (Stone and Perry (10), Hayashi (11)) bring evidences that HMM-S-1 presents a certain number of breaks in the heavy polypeptide chain. It may be mentioned that this phenomenon was also implicit in some details reported by Lowey (2). All the non dissociating techniques i.e. ultracentrifugation (Perry (10), this study), gel filtration, osmometry (Hayashi (11)) give a molecular weight for HMM-S-1 in the range 105 000 - 133 000 daltons suggesting that the neoformed fragments are held together by non covalent forces in a structure which retains at least in part the native functional characteristics. With papain and in different conditions Cooke (12) reported recently a non fragmented HMM-S-1 preparation obtained from a direct digestion of myofibrils. In this method it is expected that the native structure will provide a protection for the actin binding site, a notion which had been used earlier by Huriaux (13) to prepare HMM-S-1 from cow carotid.

Compared to Cooke's our EDTA-ATPase specific activity is low (3.2 μmol.min^{-1} mg^{-1} vs 6 μmol.min^{-1} mg^{-1}) and this might be related to the occurrence of heavy and/or light chain fragmentation although it is not possible to decide whether some molecules have suffered a type of alteration destroying the ATPase activity without impairment of the affinity for actin (since they are found in the acto HMM-S-1 complex), or all the molecules are so modified that their activity is generally lowered.

Our chromatographic enrichment definitely suggests that fragment 52 000 and 26 000 are parts of the same globule but the chromatographic heterogeneity and our other experimental data in their present state do not seem coherent enough to support or reject the hypothesis of two different kinds of globular heads in one myosin molecule.

Finally an interesting observation from the comparison of this and other available studies is that in spite of the different methods used by several laboratories the de novo fragmentation pattern is very similar in all preparations bringing a strong confirmation to the concept of a very limited number of rather well defined regions of the heavy chain which are susceptible to proteolytic attack. It may be hoped that a detailed analysis of these fragments will facilitate the localisation of the residues involved in the different functions of the HMM-S-1 globule.

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