The Changes of "Myosin B" during Storage of Rabbit Muscle

Part I. Physico-chemical Studies on "Myosin B."

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The differences between myosin B extracted from fresh muscle with Weber-Edsall solution and myosin B type protein from the muscle stored at 4°C post-mortem were investigated by the measurement of ATPase activity, ATP-sensitivity, salting-out curves, chromatographic patterns on triethylaminoethyl (TEAE)-cellulose, viscosity and ultracentrifugation diagrams. As compared with myosin B from fresh muscle and myosin B type protein from post-rigor muscle, myosin B type protein from rigor muscle showed relatively high values of ATPase activity, ATP-sensitivity and reduced viscosity, and moreover, was remarkably different in salting-out curves, precipitating gradually from ammonium sulfate of lower concentration than that in others. The differences between myosin B from fresh muscle and myosin B type protein from post-rigor muscle were clearly demonstrated by chromatography and sedimentation data.

Post-rigor changes of muscle associated with the increasing tenderness have been widely studied for many years, but the mechanism of the post-rigor tenderization is not well elucidated.

Proteolysis was found not to play an important part in post-mortem aging by Wierbicki et al.,¹⁾ Fujimaki,²⁾ Loker³⁾ and Dvorak.⁴⁾

Steiner,⁵⁾ Wierbicki et al.¹⁾ and Whitaker⁶⁾ came to the conclusion that changes in connective tissue did not appear to be closely associated with aging. Sarcoplasmic proteins seemed to change hardly post-mortem from our results of conventional electrophoretical analysis, but have been recently found to change considerable by Scopes and Lawrie⁷⁾ or Fujimaki and Deatherage.⁸⁾ Therefore, changes in sarcoplasmic proteins deserve some

attention, though they do not seem to make so drastic effect on tenderness. It might be possible to explain tenderization by changes in contractile proteins. Though actomyosin formed during rigor development was expected to dissociate into actin and myosin by Weinberg and Rose,⁹⁾ our previous report¹⁰⁾ showed that the content of extracted myosin A type protein decreased during rigor development and did not increase even after resolution of rigor, while that of extracted myosin B type protein from the muscle previously washed free from myosin A type protein increased post-mortem. Accordingly, it will be interesting to study the differences in properties of myosin B type protein extracted from muscle post-mortem. To elucidate post-mortem changes of myosin B type protein, the author¹⁰⁾ differentiated the protein into two types, actomyosin-S and actomyosin-L. It was shown in our previous report¹¹⁾ that ATPase activity, reduced viscosity

1) E. Wierbicki, L. E. Kunkle, V. R. Cahill and F. E. Deatherage, *Food Technol.*, **8**, 506 (1954).

2) M. Fujimaki, *J. Agr. Chem. Soc. Japan*, **32**, 689 (1958).

3) R. H. Locker, *J. Sci. Food Agr.*, **11**, 520 (1960).

4) Z. Dvorak, *Coll. Czech. Commun.*, **25**, 2059 (1960).

5) G. Steiner, *Arch. Lebensm.-Hyg.*, **121**, 193 (1939).

6) J. R. Whitaker, *Advances in Food Research*, **9**, 1 (1959).

7) R. K. Scopes and R. A. Lawrie, *Nature*, **197**, 1202 (1963).

8) M. Fujimaki and F. E. Deatherage, *J. Food Sci.*, **29**, 316 (1964).

9) B. Weinberg and D. Rose, *Food Technol.*, **14**, 376 (1960).

10) M. Fujimaki and H. Nakajima, *J. Agr. Chem. Soc. Japan*, **32**, 695 (1958).

11) M. Fujimaki and N. Arakawa, *This journal*, **22**, 249 (1958).

and ATP-sensitivity of both actomyosin-S and -L from stored rabbit muscle took the maximal values at the time of rigor.

This report shows the remarkable differences in salting-out curves between myosin B type proteins prepared with Weber-Edsall solution from the muscle stored for various periods, in addition to the results of the ultracentrifugal analyses, ATPase activity and viscosity. Authors¹²⁾ have found that myosin B could be fractionated by triethylaminoethyl(TEAE)-cellulose column chromatography. The differences in chromatographic patterns between myosin B type proteins from stored rabbit muscle are also investigated.

EXPERIMENTAL

Preparations of Myosin B

According to the conventional method¹³⁾ minced and stored rabbit muscle (*Longissimus dorsi*) was extracted for 24 hours with 6 volumes of Weber-Edsall solution. The brei was centrifuged and diluted to the concentration of 0.2 M of KCl with water. After the dilution, the precipitate occurred was collected by the centrifugation and the sediment was dissolved in 0.6 M KCl. The protein was refined by this dilution method three times successively. As mentioned above, myosin B was extracted from rabbit muscle immediately after slaughter (In the Tables and Figures, this has been shown as the muscle stored for 0 days.) and myosin B type protein, "myosin B", by the same method from the muscle stored at 4°C for definite days post mortem. Muscle stored for 2 or 3 days was used as the muscle sample at the step of rigor and one stored for 7 days, as that at the resolution of rigor. All preparations were done at 4°C.

ATPase Activity

ATPase activity was estimated as previously reported.¹¹⁾ The reaction mixture (8 ml) composed of protein of final concentration about 0.05 mg/ml, 1 mM MgCl₂ or CaCl₂, 1 mM ATP, 0.01 M tris-chloride buffer (pH 8.0) and the ionic strength adjusted with KCl, was incubated at 25°C for 5 minutes. Liberated phosphorus was measured by the modified Allen's method.¹⁴⁾

12) N. Arakawa, A. Okitani and M. Fujimaki, This journal, 29, 574 (1965).

13) A. Szent-Gyorgyi, "Chemistry of Muscular Contraction," Academic Press, New York (1951).

14) M. Nakamura, *J. Agr. Chem. Soc. Japan*, 24, 1 (1950).

ATP-sensitivity

APT-sensitivity was measured as previously reported.¹¹⁾

Salting-out

Principally the method of Tenow and Snellmann¹⁵⁾ was followed: A series of 0.1 M K-phosphate buffer solution containing 0.5 M KCl and increasing amounts of ammonium sulfate (pH 7.0) was prepared. To 3.0 ml of this solution, 2 ml of protein solution (ca. $E_{1cm}^{1.0} = 1.0$ at 280 m μ) was added and the mixture was allowed to stand at 4°C for about 18 hours. Then it was centrifuged at 2,500 g for 5 minutes and the amount of protein in the supernatant was determined by measuring the light absorption at 280 m μ .

Chromatography

Column chromatography of myosin B on TEAE-cellulose has been described in the previous paper.¹²⁾ Protein was applied to the column of TEAE-cellulose bufferized with 0.02 M tris-chloride buffer containing 0.28 M KCl (pH 8.0) and eluted with the same buffer, 0.02 M tris-chloride buffer containing 1.0 M KCl (pH 8.0), and 0.5 N NaOH stepwisely at 4°C. The amount of protein eluted was determined by the measurement of the light absorption at 280 m μ .

Viscosity

Viscosity of the protein in 0.02 M tris-chloride buffer containing 0.28 M KCl (pH 8.0) was measured with an Ostwald viscometer at 25°C.

Ultracentrifuging

Ultracentrifuging was carried out with Hitachi UCA-1A at about 20°C. The sedimentation coefficient, $s_{20,w}$ was calculated by the schlieren diagrams.

Protein Concentration

Protein concentration was determined by semi-micro-Kjeldahl method, using a factor of 6.0 in the experiment of ATPase activity, viscosity and ultracentrifuging.

RESULTS

Extractability

In previous report,¹⁰⁾ it has been shown that myosin B type protein extracted from stored veal muscle with 0.6 M KCl solution and Weber-Edsall solution respectively increased gradually post-mortem. "Myosin B" extracted with Weber-Edsall solution from stored rabbit muscle increased with the progress of storage

15) M. Tenow and O. Snellmann, *Biochim. Biophys. Acta*, 15, 395 (1954).

TABLE I. AMOUNTS OF "MYOSIN B" EXTRACTED WITH WEBER-EDSALL SOLUTION FROM STORED RABBIT MUSCLE

Storage period (day)	Protein conc. of solution (mg/ml)	Volume of solution (ml)	Total protein content (mg %)
0*	5.43	93	920
2	5.09	115	1,140
7	4.62	140	1,290

* This means myosin B, as shown in the text (page 582).

period as shown in Table I. Therefore, Weber-Edsall solution seemed to be effective to extract almost all the main contractile proteins that were supposed to take important parts during development and resolution of rigor.

ATPase Activity and ATP-sensitivity

In our previous paper, ATPase activity of actomyosin-S and -L was found to be maximal at rigor, as measured in the presence of Ca^{++} at high ionic strength ($I=0.6$). ATPase activity of myosin B in the presence of Ca^{++} was found to be activated at optimal ionic strength between 0.10 and 0.15 by Maruyama.¹⁶⁾ The difference in ATPase activity between "myosin B" proteins in the presence of Ca^{++} at low ionic strength ($I=0.14$) was similar to that in ATPase activity between proteins of actomyosin-S or -L¹¹⁾ respectively (Table II). The Ca -activated-ATPase activity of "myosin B" from the muscle stored for 2 days was 1.2~1.4 times that of myosin B, whereas that of "myosin B" from the muscle stored for 7 days was about 0.75 times that

of myosin B. As ATPase of myosin A is also activated by Ca^{++} , it may be necessary in this case to consider the contamination of myosin A into the preparations of myosin B (see discussion).

The enhancement of ATPase activity of myosin B by Mg^{++} is specific at low ionic strength ($I=0.035$),¹⁶⁾ but that of myosin A is not activated by Mg^{++} at any ionic strength. Therefore, it was considered that the ATPase activity of myosin B preparation measured in the presence of Mg^{++} at $I=0.035$ was attributed to myosin B alone (Table II). Mg -activated-ATPase activity of "myosin B" from the muscle stored for 2 days was relatively high, 1.2~1.3 times that of myosin B, and "myosin B" from the muscles stored for 7 days was not less than that of myosin B.

TABLE III. ATP-SENSITIVITY OF "MYOSIN B" FROM STORED RABBIT MUSCLE.

Protein concentration; $E_{1\text{cm}}^{2.5}$ at 280 $m\mu$ in 0.28 M-KCl-0.02 M tris-chloride (pH 8.0), MgCl_2 ; 1 mM, ATP; 1 mM, Temp.; 4°C

Storage period (day)	ATP-sensitivity (%)
0	63.1
2	86.8
7	76.2

Higher ATP-sensitivity was shown in "myosin B" from the muscle stored for 2 days than in myosin B and "myosin B" from the muscle stored for 7 days, as presented in Table III.

Salting-out

Sasaki¹⁷⁾ reported that a main peak in the salting-out curve of rabbit myosin B was observed at 28 to 32% ammonium sulfate saturation and another small peak which seemed to correspond with myosin A was at 33 to 34%. The authors¹¹⁾ observed that the concentration of ammonium sulfate in salting-out of actomyosin-S and -L from horse and veal muscle shifted with the aging of meat.

The investigation on differences in salting-out curves between "myosin B" obtained from

TABLE II. ATPASE ACTIVITY OF "MYOSIN B" FROM STORED RABBIT MUSCLE.

The condition of reaction is shown in the text.

KCl conc.	Added metal	Sample	ATPase activity (Pi μg /min/mg protein/ml storage period (day))		
			0	2	7
0.035	Mg	A	30.6	35.4	31.0
		B	25.1	31.8	30.9
0.14	Ca	A	25.1	34.8	19.2
		B	25.5	31.2	19.1

16) K. Maruyama, *Biochim. Biophys. Acta*, **77**, 682 (1963).

17) A. Sasaki, *J. Research Inst. Catalysis*, **6**, 221 (1958).

FIG. 1. Differential Salting-Out Curves of "Myosin B" (Sample B) from Stored Rabbit Muscle.

C is % saturation of $(\text{NH}_4)_2\text{SO}_4$. S is solubility %.

○—○, 0 day; ▲—▲, 2 days; ●—●, 7 days

stored rabbit muscle (Fig. 1) showed that myosin B had a main component which was precipitated at 34 to 37% ammonium sulfate saturation and a minor component at 38 to 41%. The discrepancy may be due to the difference in the experiment method. In some range, the supernatant added with ammonium sulfate showed higher extinction than that of the control solution, owing to turbidity approaching near the precipitating region of the protein. The curve of "myosin B" from the muscle stored for 2 days differed remarkably from that of myosin B and "myosin B" from the muscle stored for 7 days. The precipitation being distributed in a wide range of the concentration of ammonium sulfate, the main peak consequently was small. The curve of "myosin B" from the muscle stored for 7 days was considerably similar to that of myosin B, but its major component was precipitated at a slightly lower concentration of ammonium sulfate, as compared with that of myosin B, followed by the minor component. Both "myosin B" showed a small peak which corre-

sponded to myosin A peak presented in Fig. 1.

Chromatography

The behaviour of "myosin B" from stored muscle on TEAE-cellulose column was investigated. The chromatographic patterns obtained are shown in Fig. 2. As shown in Table IV, the protein content of the corresponding peaks varied considerably from sample C to sample D. However, that of the first eluted peak in myosin B was always larger than that of the secondly eluted peak.

TABLE IV. PROTEIN CONTENT OF EACH PEAK IN "MYOSIN B" FROM STORED RABBIT MUSCLE ON TEAE-CELLULOSE COLUMN CHROMATOGRAPHY

Peak No.	Sample storage period (day)	Protein content (% applied protein)					
		C*			D		
		0	3	7	0	2	7
1		54.3	30.6	17.1	36.9	15.3	9.8
2		21.8	32.4	38.4	32.2	45.3	40.3
3		none	4.2	5.4	3.2	6.5	2.8
4		25.8	23.9	17.4	35.1	27.9	20.9
recovery		101.9	91.1	78.3	107.4	95.0	73.8

* Chromatogram is shown in Fig. 2.

FIG. 2. Chromatography on "Myosin B" (Sample C) from Stored Rabbit Muscle on TEAE-Cellulose.

Protein solution (ca. 40 ml.: $E_{1\text{cm}}$ ca. 3 at $280 \text{ m}\mu$) in 0.28 M-KCl-0.02 M-tris-HCl (pH 8.0) was applied to a column $20 \text{ cm} \times 3.5 \text{ cm}$ diam. Step to 1.00 M-KCl-0.02 M-tris-HCl at the point marked A and to 0.5N NaOH at B. Curves from top to bottom are: 0 day; 3 days; and 7 days.

The difference in the protein content of each peak between myosin B and "myosin B" from the muscle stored for 2 or 3 days may be related with changes in contractile proteins during rigor development. The decrease of the protein content of the first peak in "myosin B" from the muscle stored for 7 days may be related with changes in contractile proteins during resolution of rigor, though the change in the protein content of the second peak did not show the same tendency in the case of sample C and D. The remarkable difference in chromatograms between "myosin B" from the muscle stored for 7 days and that for 2 days was that both the first and the second peaks in the former showed less sharp and broader patterns, and thus more poly-disperse than those in the latter.

Two peaks were always eluted with 0.5N NaOH solution after the elution of the first and the second peaks except in the case of myosin B (sample C) in which only one peak was eluted. As these peaks always show higher extinction at 260 m μ than at 280 m μ , they may contain relatively large amount of ribonucleotides. Changes in the protein con-

tent of these two peaks between "myosin B" from stored muscle did not show the same tendency in the case of sample C and D, suggesting little relation with changes in contractile proteins during development and resolution of rigor.

The recovery of the protein applied decreased gradually with the progress of storage period. This seemed to relate with denaturation of protein post-mortem.

Viscosity

It is well known that the viscosity of myosin B is remarkably dependent on velocity gradient of flow. The intrinsic viscosity should be measured at low velocity gradient of flow and low concentration of protein. However some information on difference in the axial ratios of protein molecules will be given by the measurement of the viscosity at such high velocity gradient of flow as in this experiment (Fig. 3). The reduced viscosity of "myosin B" from the muscle stored for 7 days was lower than that of myosin B within all ranges of concentration measured. The higher viscosity of

FIG. 3. Concentration Dependence of Reduced Viscosity of "Myosin B" (Sample A) from Stored Rabbit Muscle.

○, 0 day; ▲, 2 days; ●, 7 days

FIG. 4. Sedimentation Diagram of Rabbit Myosin B.

Total protein concentration, 3 mg/ml in 0.6M-KCl-0.02M-tris-HCl (pH 7.2), 28°C. Speed, 51,200 r.p.m.; bar angle, 50°; 12 min after reaching top speed.

FIG. 5. Sedimentation Diagram of "Myosin B" (Sample A) from Stored Rabbit Muscle.

Total protein concentration, 3 mg/ml in 0.28 M-KCl-0.02 M-tris-HCl (pH 8.0), speed, 40,370 r.p.m.; bar angle, 50°; 6 min after reaching top speed. (a) 0 day, 22°C; (b) 2 days, 23°C; (c) 7 days, 25°C.

"myosin B" from the muscle stored for 2 days may explain that its axial ratio is larger than those of myosin B and "myosin B" from the muscle stored for 7 days.

Ultracentrifugation

The sedimentation diagram of myosin B at the concentration of 3 mg per ml showed two main peaks of 22 S and 25 S in 0.6 M KCl, whereas two main peaks of 50 S and 52 S in 0.28 M KCl (Fig. 4 and 5a).

For very elongated molecules, the sedimentation coefficients of the side by side polymers of n -fold monomers are shown to be n times that of the monomers.¹⁸⁾ Hence, myosin B molecules in 0.28 M KCl are supposed to be side by side aggregates of several monomeric myosin B molecules in 0.6 M KCl.

The sedimentation behavior of "myosin B" from stored muscle was studied in the concentration of 0.28 M KCl in order to know the correlation with the results of chromatography and viscosity. The sedimentation diagrams of "myosin B" from the muscle during storage were shown in Fig. 5. Though it was difficult

to know the accurate concentration of each peak by the measurement of its area on the diagram because of the abnormal sharpening of the peak, the sector-dilution effect and Johnson and Ogston's effect,¹⁹⁾ results of rough calculation revealed that the concentration ratio of the two main peaks in each diagram was not so different. The remarkable difference in the shape of the rapid peaks between "myosin B" from the muscle stored for 2 days and myosin B may suggest changes in molecular shape and size of contractile proteins during rigor development, but further studies will be necessary for the details. The sedimentation diagram of "myosin B" from the muscle stored for 7 days was not so different from that of "myosin B" from the muscle stored for 2 days. Further experiments of ultracentrifugation at various protein concentrations may reveal the difference in the molecular width of each peak component.

DISCUSSION

Higher values in ATPase activity and ATP-

18) H.H. Weber and H. Portzehl, *Advances in Protein Chem.*, 7, 211 (1952).

19) J.P. Jonston and A.G. Ogston, *Trans. Faraday Soc.*, 42, 789 (1946).

sensitivity of "myosin B" from the muscle stored for 2 days revealed that it was composed mainly of intact myosin B arranged differently from that from fresh muscle, with some denatured myosin B fraction. This arrangement in myosin B molecules seemed to be associated with changes in contractile proteins in muscle at the step of rigor. This arranged myosin B obviously corresponded to the proteins precipitating at lower concentration of ammonium sulfate in the salting-out curve of "myosin B" from the muscle stored for 2 days. F-actin was reported to be precipitated at 10~30% saturation of ammonium sulfate by Sasaki.¹⁷⁾ Therefore, these precipitated proteins could be myosin B rich in F-actin, and were relatively lacking in free charge, which seemed to bring about partly lack of hydration which was supposed to have some effect on the poor water-holding capacity of rigor muscle.

The higher axial ratio of "myosin B" from the muscle stored for 2 days by the results of reduced viscosity may also suggest that this arranged myosin B was constructed with relatively long F-actin threads. When more sedimentation data are added, the relative differences in molecular length and width will be elucidated.

This arranged myosin B also seemed to correspond to the increment of the proteins eluted at the second peak in the chromatography of "myosin B" from the muscle stored for 2 or 3 days. Further investigation for each peak may help to elucidate the properties of this arranged myosin B.

It was difficult to elucidate whether the difference of ATPase activity and ATP-sensitivity between "myosin B" from the muscle

stored for 2 days and that for 7 days was caused by any rearrangement associated with resolution of rigor in the myosin B molecules once formed during rigor development or/denaturation during storage. However, the decrement of Ca-activated-ATPase activity was evidently caused mainly by the denaturation of myosin A type proteins contaminating in myosin B type protein preparations, because Mg-activated-ATPase activity which was specific for myosin B type proteins did not decrease so much.

The results of viscosity and salting-out showed that "myosin B" from the muscle stored for 7 days was remarkably different from that stored for 2 days, and seem to suggest that the arranged myosin B molecules at the step of rigor was again rearranged during resolution of rigor. From the results of salting-out, viscosity, ATPase activity and ATP-sensitivity, this rearrangement appeared to proceed through the reverse process of the arrangement during rigor development. However, the remarkable difference of chromatographic patterns and sedimentation diagrams between "myosin B" from the muscle stored for 7 days and myosin B from fresh muscle revealed clearly that the rearranged myosin B at any step of resolution was far more different from myosin B than from the arranged myosin B at the step of rigor.

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