Systematic Fractionation of Sarcoplasmic Proteins of Rat Skeletal Muscle

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Fractionation of sarcoplasmic proteins of various species has long been tried mainly by two groups of authors. One group has an interest in the metabolism of sarcoplasmic proteins^{1~12,14)} and the other in the post-mortem changes of muscle.^{13,19~21,23,24)} Several techniques of protein fractionation have been applied; ammonium sulfate,^{2,5)} acetone,⁷⁾ free boundary electrophoresis,^{3,4,6)} chromatography on ion exchange celluloses^{10,14,15,20,21,23)} and starch,^{9,11~14,16,18,19,22,24)} acrylamide or agar gel electrophoresis. Especially, by using ion exchange celluloses or gel electrophoresis, more than 10 components have been identified as the components of sarcoplasmic proteins.

However, systematic fractionation of sarcoplasmic proteins by combination of several techniques seems not to have been fully attempted. In the present investigation, systematic fractionation of sarcoplasmic proteins of rat skeletal muscle by applying ammonium sulfate fractionation, CM-cellulose and DEAE-cellulose chromatography has been attempted. The fractions obtained were identified and their purity was checked by means of starch gel electrophoresis.

Animals. Adult male Wistar strain albino rats were used throughout the experiments. Animals were fed on the stock diet of this laboratory²⁵⁾ containing 76% wheat, 15% fish meal, 5% alfalfa meal, 2% soybean oil, 1% dried brewer's yeast, 0.7% calcium carbonate, and 0.3% sodium chloride.

Preparation of sarcoplasmic proteins. Sarcoplasmic proteins were prepared according to Hartshorne and Perry,¹⁴⁾ as follows: Rats were lightly anesthetized with ether and slaughtered by exsanguination. Hind limbs were quickly excised and chilled in ice cold homogenizing solution (20 mM tris-50 mM KCl-HCl, pH 7.6). Thereafter, the limbs were blotted and muscles were separated from bones. Large connective tissues and fat pads were removed. Muscles were cut into small pieces by a pair of scissors and homogenized in a Waring blender-type homogenizer with a volume of the homogenizing solution 3 times their weight. The homogenate was centrifuged for 20 min at $10,000\times g$ and the supernatant was used as the sample proteins.

Ammonium sulfate fractionation. Solid ammonium sulfate was added to the sarcoplasm until the saturation level indicated in the figure was attained, keeping pH of the solution from 6.0 to 6.3 by adding solid Na_2HPO_4 . The precipitate was centrifuged and dissolved in 40 mM glycine-NaOH (pH 9.1) buffer.

Column chromatography on ion exchange cellulose. CM-cellulose and DEAE-cellulose were treated on a filter alternately with 0.5N HCl and 0.5N NaOH, and they were washed with distilled water between and after each treatment until the washings reached near neutral range. DEAE-cellulose was first treated with acid and then alkali, whereas the reverse

FIG. 1. Starch Gel Electrophoresis of Sarcoplasm of Rat Skeletal Muscle.

- 1 a: Whole sarcoplasm stained by Amido Black 10B.
 1 b: Whole sarcoplasm stained by Oil Red O.
 1 c: Whole sarcoplasm stained by benzidine.
 1 d: Sarcoplasmic proteins precipitated at 0~50% saturation of $(\text{NH}_4)_2\text{SO}_4$.
 1 e: Sarcoplasmic proteins precipitated at 50~60% saturation of $(\text{NH}_4)_2\text{SO}_4$.
 1 f: Sarcoplasmic proteins precipitated at 60~70% saturation of $(\text{NH}_4)_2\text{SO}_4$.
 1 g: Sarcoplasmic proteins soluble at 70% saturation of $(\text{NH}_4)_2\text{SO}_4$.

■ Strongly stained zones
 ▨ Weakly stained zones
 □ Faintly stained zones
 ☼ Stained parts not showing clear zones

FIG. 2. CM-cellulose Chromatography of Sarcoplasmic Proteins.

The following buffers were used.

Buffer solution a: 5 mM KH_2PO_4 -NaOH pH 6.1

Buffer solution b: 5 mM Na_2HPO_4

Starting buffer; a : b = 10 : 0

After point A; a : b = 8 : 2 plus KCl to 20 mM

After point B; a : b = 5 : 5 plus KCl to 50 mM

After point C; a : b = 3 : 7 plus KCl to 100 mM

After point D; a : b = 0 : 10 plus KCl to 300 mM

Optical density at 260 $m\mu$ is not shown except a part of peak 1, because abnormal absorption was not found.

Fig. 2a: Whole sarcoplasm as indicated in Fig. 1a Fig. 2c: Starch gel electrophoresis of peak 3

Fig. 2b: Starch gel electrophoresis of peak 1

Other peaks were not examined because of low yield of proteins.

○---○ O.D. at 280 $m\mu$, ●---● O.D. at 260 $m\mu$, ●—● O.D. at 410 $m\mu$

FIG. 3. Captions put into the bottom of the next page.

FIG. 4. Starch Gel Electrophoresis of Rat Sarcoplasmic Proteins and Serum Proteins.

Fig. 4a: Sarcoplasmic proteins as indicated in Fig. 1 a.

Fig. 4b: Serum Proteins.

FIG. 5. Starch Gel Electrophoresis of Sarcoplasmic Proteins of Normal and Protein Depleted Rats.

Fig. 5a: Normal.

Fig. 5b: Protein depleted.

Normal diet³⁵⁾ contained 65.6% corn starch, 20% whole egg protein, 5% soybean oil, 5% cellulose powder, 4% salt mixture, 0.3% vitamin mixture, and 0.1% choline chloride. Protein free diet contained 0% whole egg protein and 85.6% corn starch, and other components were the same as that of normal diet. Protein was depleted by giving the protein free diet for 16 or 32 days.

FIG. 3. DEAE-cellulose Chromatography of Sarcoplasmic Proteins.

Starting buffer: 40 mM glycine-NaOH pH 9.1.

After point A; KCl was added to the buffer to 10 mM.

After point B; KCl was added to the buffer to 30 mM.

After point C; KCl was added to the buffer to 100 mM.

After point D; KCl was added to the buffer to 200 mM.

Optical density at 260 m μ is not shown except a part of the chromatogram, because abnormal absorption was not found.

Fig. 3a: Whole sarcoplasm as indicated in Fig. 1 a and Fig. 2a.

Fig. 3b: Starch gel electrophoresis of peak 1.

Fig. 3c: Starch gel electrophoresis of peak 2.

Fig. 3d: Starch gel electrophoresis of peak 3.

Fig. 3e: Starch gel electrophoresis of peak 4 and 5.

Fig. 3f: Starch gel electrophoresis of peak 6.

As for the symbols, see Fig. 2.

procedure was used for CM-cellulose. Each cellulose was then equilibrated with the starting buffer. Sarcoplasmic proteins were dialyzed to the starting buffers and 30 ml of dialyzates were applied to a column (3×13 cm). In the case of CM-cellulose chromatography, dialysis of sarcoplasmic proteins against 10 mM phosphate buffer brought about partial precipitation of proteins. The precipitate was removed by centrifugation and the supernatant was used as the samples of the chromatography.

One hundred and fifty drops of eluate each were collected and the optical densities at 280 m μ , 260 m μ , and 410 m μ (colored fractions) were measured. To the fractions of each peak were added solid ammonium sulfate until the saturation was attained. The precipitate was collected by centrifugation and dissolved in a small volume of 40 mM glycine-NaOH (pH 9.1) buffer in order to apply to the starch gel electrophoresis. DEAE-cellulose chromatography was performed according to Hartshorne and Perry.¹⁴⁾

Starch gel electrophoresis. Vertical starch gel electrophoresis was done according to Smithies,^{26,27)} partially modified by Aoki.²⁸⁾ The size of gel tray was 6×60×260 mm. One per cent of sodium chloride was used as the electrode solution and 110 mM boric acid-NaOH (pH 8.8) as the bridge solution. Electrophoresis was performed for 16 hr at the constant flow of 5 mA. Proteins were stained with Amido Black 10B, lipid containing fractions with Oil Red O, and colored proteins with benzidine, according to Smithies.²⁷⁾

The electrophoretic pattern of whole sarcoplasm is shown in Fig. 1a. More than 16 zones were identified and numbered from anode to cathode. Large part of the proteins seems to be localized continuously between zone 7 and the original point. This part was designated as zone 9 tentatively. Since zone 3, 11, 12 and 13 were also stained by Oil Red O, these zones seem to contain lipoproteins (Fig. 1b). Figure 1c shows the benzidine posi-

tive zones. At least 3 zones were stained by this method, but they did not coincide with any zones of Amido Black 10B staining. This result is in accordance with that of Laurent *et al.*²⁹⁾ and probably with that of Formanek *et al.*³⁰⁾ Figures 1d, e, f and g show the electrophoretic patterns of fractions obtained by ammonium sulfate fractionation. Many zones were recovered in more than two fractions by this fractionation, except zone 7 and colored proteins which were almost exclusively recovered in the supernatant of 70% saturation.

Figure 2 shows the chromatographic pattern of sarcoplasmic proteins on CM-cellulose. The electrophoretic pattern of the fractions obtained by CM-cellulose chromatography is shown in Fig. 2b and c. More cathionic components than zone 8 were recovered in peak 3 and others were obtained in peak 1. By this chromatography, colored proteins were recovered mainly in peak 2. This method did not give good fractionation. One of the reasons for this result seems to be that CM-cellulose was used at pH 6.1. Below pH 6.0, many sarcoplasmic proteins precipitate, and, on the contrary, CM-cellulose does not function well above pH 6.0. In spite of these defects, CM-cellulose chromatography brought about clear-cut separation of zone 3 and 7 from other main zones.

Figure 3 shows the result of DEAE-cellulose chromatography, by which the sarcoplasmic proteins were fractionated into 6 main fractions. The electrophoretic patterns of the fractions are shown in Fig. 3b, c, d, e and f. By this chromatography, components of sarcoplasmic proteins were eluted in order from cationic ones to anionic ones. Peak 6 contains zone 3 almost exclusively. The peak appeared between peak 5 and 6 showed stronger absorption at 260 m μ than at 280 m μ , which suggests this fraction to be nucleic acid(s) or the related compound(s). Colored proteins were recovered mainly in the peaks 2 and 3, but small portions were recovered also in the peak 4. This result might suggest the heterogeneity of

myoglobins³⁰⁻³²⁾ or coexistence of myoglobin, hemoglobin and their derivatives.

The problem whether or not the zones identified by starch gel electrophoresis are of muscle cell origin or serum origin, was examined. Figure 4 shows the result of this experiment. This result indicates that main zones of sarcoplasmic proteins are of muscle cell origin except zone 3. Zone 3, which presumably corresponds to myoalbumin described by Bates-Smith³¹⁾ and by Jacob,⁴¹⁾ may or may not be serum protein origin as discussed by Gitlin *et al.*^{33,34)} and by Hartshorne *et al.*¹⁴⁾ This problem remains to be elucidated.

Another problem whether or not protein depletion affects the electrophoretic pattern of sarcoplasmic proteins was further examined. The result is shown in Fig. 5 which suggests no effect of feeding protein-free diet on any zone except zone 3. Zone 3 seems to decrease in the protein depleted animals. As serum

albumin has been reported to decrease in protein depleted animals,³⁶⁻³⁸⁾ this result should be studied further in relation with the above mentioned problem.

By applying ammonium sulfate fractionation, CM-cellulose chromatography, and DEAE-cellulose chromatography, sarcoplasmic proteins of rat skeletal muscle were fractionated into several fractions. The results are summarized in Fig. 6. From Fig. 6, it is suggested that some of the sarcoplasmic proteins were obtained as a single protein, as far as it was identified on the starch gel electrophoresis. For example, zone 3 can be obtained as a highly purified preparation by applying DEAE-cellulose chromatography, and zone 7 can be obtained by ammonium sulfate fractionation followed by CM-cellulose chromatography. Other zones can also be obtained as highly purified preparations by applying these techniques, even if they are not obtained as

Zone No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	C.P.*
Fraction (NH ₄) ₂ SO ₄ 0~50%			▨								▨				▨	▨	
(NH ₄) ₂ SO ₄ 50~60%			▨	▨				▨		▨	▨	▨	▨	▨			
(NH ₄) ₂ SO ₄ 60~70%			▨			▨			▨	▨	▨		▨	▨			
(NH ₄) ₂ SO ₄ 70%~							▨			▨	▨	▨					▨
CMC peak 1			▨			▨	▨	▨									
CMC peak 2																	▨
CMC peak 3											▨	▨	▨	▨	▨	▨	
DEAEC peak 1													▨	▨	▨		
DEAEC peak 2							▨	▨				▨	▨	▨	▨	▨	▨
DEAEC peak 3								▨	▨	▨	▨						▨
DEAEC peak 4,5						▨				▨	▨						▨
DEAEC peak 6			▨														

FIG. 6. Summary of Fractionation of Sarcoplasmic Proteins.

As for symbols, see Fig. 1.

* Colored proteins

a single protein. Further studies to obtain more purified preparations systematically are now under investigation and will be published later.

These studies may give methods for elucidating the metabolic heterogeneity of sarcoplasmic proteins or for studying the effects of hormones and drugs on synthesis and degradation of each sarcoplasmic protein.

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