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In vitro stability and protein composition of thick filaments from insect flight muscles

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Abstract. Thick and thin filaments of synchronous and asynchronous insect flight muscles were separated by density gradient centrifugation. A good release of myofilaments from myofibrils was obtained by sonication of myofibrils in relaxing solution with pH 6.1 (locust), pH 6.4 (honeybee) and pH 6.6 (fleshfly), respectively. Thick filaments but not thin filaments were dissolved, if sucrose gradient centrifugation was used to separate the filaments. Thus, sucrose gradients are the medium of choice if actin filaments are to be purified. Glycerol-containing gradients selectively dissolved myosin filaments from fleshfly muscles. The stability of the myosin filaments of all muscles was sufficient in gradients with 10–30% formamide.
Key words. Insect muscle; myosin filaments; contractile proteins.

The contractile apparatus of insect flight muscles differs from that of vertebrate skeletal muscles in structure and in its regulatory mechanisms, and has a considerably higher ATP splitting rate during activity. The molecular basis of these differences, however, is not yet fully known. Careful studies on the protein pattern of actin filaments[1–3] and the Z-line[1] of insect flight muscles demonstrated the existence of insect-specific proteins. For insect myosin filaments, muscle-specific contents of paramyosin[4,5] and the existence of a high molecular weight protein connecting the myosin filaments to the Z-line[1,6,7] have been found, but M-line proteins could not yet be identified. One of the reasons for this information gap seems to be the instability of native thick filaments from insect muscles during density gradient centrifugation. The aim of this work, therefore, was to improve the purification procedures for these filaments to facilitate the analysis of their protein composition.

Flight muscles (0.5 to 1 g) of 6 locusts (*Locusta migratoria*) or whole thoraces of 40 fleshflies (*Phormia terrae-novae*) or 30 honeybees (*Apis mellifica*) were used for the preparation of myofibrils[8]. All procedures were performed at 0 to 6 °C. Filament suspensions were obtained by either a 3 × 5-s homogenization (MSE homogenizer, 15,000 rpm) or a 15-s sonication (Bandelin Sonorex–Rapid GR 80, 40 kHz, 80/320 W) of myofibrils in 3 ml relaxing buffer (10 mM imidazole or 10 mM morpholinoethane sulfonic acid = MES, 10 mM KH_2PO_4, 10 mM $MgCl_2$, 10 mM ATP, 100 mM KCl, 2 mM EGTA, 1 mM DTE) per g muscle. Sonication or homogenization were followed by 10 min centrifugation at 20,000 × g. Another two sonication/homogenization and centrifugation cycles were run with the sedimented myofibrils. The supernatants were collected and assembled. In spite of extensive fragmentation of the filaments by the sonication procedure (fig. 1) this method was used because it gave a higher yield of free filaments.

250 µl of the filament suspension ($E_{310} \leq 1.0$) with 5% of the density medium was layered on top of 13 ml of a linear density gradient[9] and overlayered with 750 µl of the filament suspension. This system was centrifuged in a Sorvall OTD-65 swinging bucket rotor at 68,000 × g for 150 min. Eight equal-volume fractions were withdrawn from the bottom of the tube with the aid of a glass capillary and a peristaltic pump[9]. Separation of thick and thin filaments was checked by taking samples from individual fractions for negative staining (1% uranylacetate in H_2O) and electron microscopy (Zeiss EM 9) and by centrifuging the rest of the fractions for 2 h at 120,000 × g and using the sediment for SDS polyacrylamide gel electrophoresis (7.5% gels, Tris · HCl[9,10]).

To exclude an influence of endogenous proteases on the results of gel electrophoresis as far as possible, different protease inhibitors, such as leupeptin (Serva, 1 mg/l), Pepstatin A (Fluka, 10^{-6} M), phenylmethylsulfonyl fluoride (Serva, 0.1 mM), soybean trypsin inhibitor (Serva, 25 mg/l) and tosyl-L-phenylalanine chloromethyl ketone (Fluka, 20 mg/l) were added alone or in combination to the solutions during the preparation of myofibrils. No differences in the SDS gel pattern of myofibrillar proteins

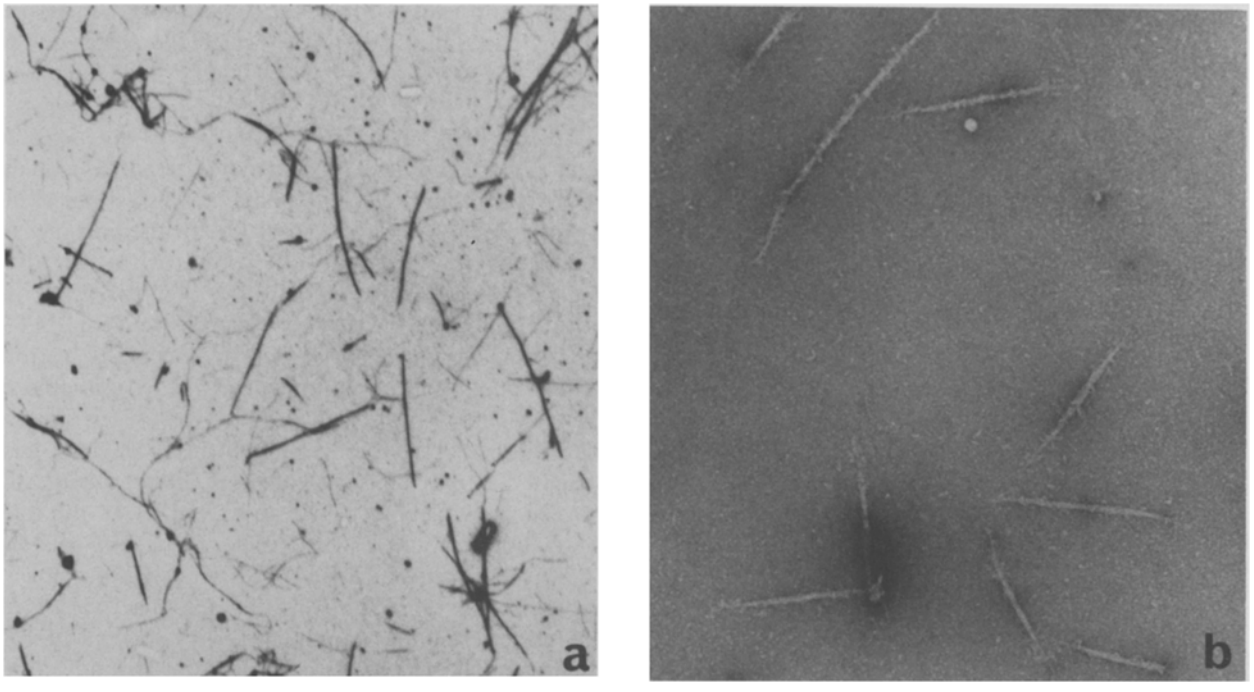

Figure 1. Negatively stained filament preparations from locust flight muscles before (*a*) and after (*b*) density gradient centrifugation (10 to 30% formamide). (*a*) shows actin and myosin filaments of a filament suspension which was obtained by homogenization with an MSE homogenizer (magnification 6400 ×). (*b*) shows the filaments of the fraction at the bottom of the tube after a density gradient run. The filament suspension layered on top of the gradient has been obtained by sonication. Only fragments of myosin filaments but no actin filaments are seen (magnification 46,000 ×).

could be observed whether protease inhibitors were present during the preparation or not. Therefore, these patterns of myofibrillar proteins were used as reference (fig. 3). It is not certain, however, whether protein bands observed in the patterns of purified myosin or actin filaments can indeed be considered to be genuine proteins. The bands might also have been caused by products of the in vivo metabolism of the contractile proteins[12].

The yield of insect myosin filaments strongly depends on the pH of the relaxing buffer. Myofibrils were suspended in relaxing buffers withs pHs of 5.5, 5.7, 5.9 (MES), 6.1, 6.3, 6.4, 6.6, or 6.8 (imidazole), homogenized and centrifuged as described above. The supernatant was applied to carbon-coated grids, negatively stained and analyzed in the electron microscope. The best yield of filaments was achieved at pH 6.1 with locust muscles (fig. 1), at pH 6.4 with honeybee muscles and at pH 6.6 with fleshfly muscles. These optimal pH values were used in the following experiments. But even then, the amount of filaments obtained varied with the kind of muscle used. The preparation of the synchronous flight muscles of 6 locusts gave about 34 mg of actin and myosin filaments which could be used for density gradient centrifugation. The asynchronous flight muscles of 30 honeybees and 40 fleshflies gave only 7 mg and 4 mg, respectively.

The stability of the myosin filaments during density gradient centrifugation seems to depend on the medium used for the density gradient. If gradients containing 2.5% to 20% sucrose were used to separate actin and myosin filaments[4], pure actin filaments but no thick filaments were found in the fractions. Myosin was present in high amounts together with actin in the top fraction, and as bulk material at the bottom of the tube. Sucrose can, therefore, not be used as supporting medium if insect myosin filaments are wanted. Sucrose is, however, the

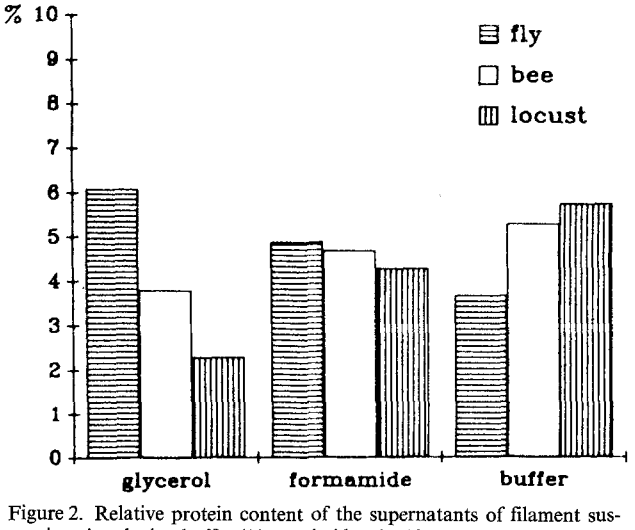

Figure 2. Relative protein content of the supernatants of filament suspensions in relaxing buffer (10 mM imidazole, 10 mM KH_2PO_4, 10 mM $MgCl_2$, 10 mM ATP, 100 mM KCl, 2 mM EGTA, 1 mM DTE) without gradient medium (= buffer) or with 35% glycerol or 30% formamide after centrifugation for 2 h at 120,000 × g. The protein content of each supernatant was measured with the Bradford test[11] and given as a percentage of the content of the whole suspension. The protein compositions of the supernatants were analyzed using SDS gels (fig. 3).

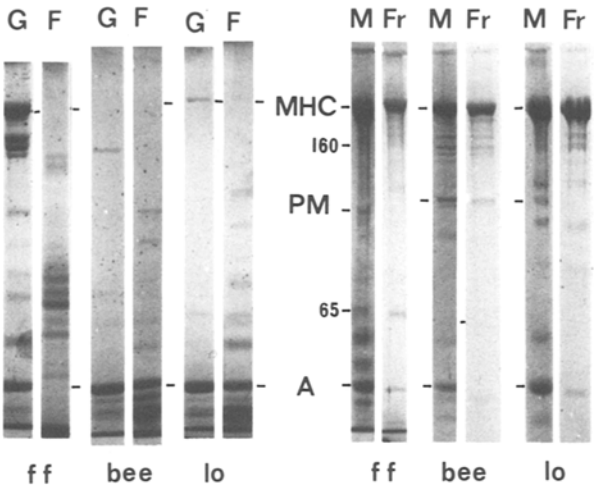

Figure 3. 7.5% SDS gels with proteins of the supernatants of centrifuged filament suspensions in glycerol (G) and formamide (F) solutions (see fig. 2) are seen at the left side. Right side: protein patterns of myofibrils or of isolated myosin filaments (bottom fractions of formamide gradients) from locust (lo), honeybee (bee) or fleshfly (ff) flight muscles. The photographs at the left side of the figure are taken from the same gel, the 3 pairs of photographs on the right are from 3 different gels. MHC = myosin heavy chain, A = actin, PM = paramyosin, M = myofibrils, Fr = fractions of myosin filaments. The calculated apparent molecular weights of other bands are given in kD.

medium of choice if pure actin filaments are to be analyzed[4].

To get myosin filaments, therefore, other media had to be tested, such as glycerol[9], and formamide which is similar to glycerol in density and has a dielectric constant similar to that of water. The stability of the filaments was examined by electron microscopy and by estimation of the loss of protein in the supporting medium. For this examination, separated actin and myosin filaments in relaxing buffer were spun down for 2 h at 120,000 × g, resuspended in relaxing buffer with 35% glycerol or 30% formamide and incubated at 4 °C for 2.5 h. One sample in relaxing buffer was allowed to stand for 4.5 h. All samples were then centrifuged for 2 h at 120,000 × g. The loss of protein of the filaments was examined by measuring the protein concentration in the supernatant. Fig. 2 shows that 2–6% of protein is lost independently of the medium used in the test, even in relaxing buffer. The largest differences appeared in glycerol solution: the locust filaments seem to be the most stable, the fleshfly filaments the least stable ones. The protein patterns of the supernatants (fig. 3, left side) show that, in the case of fleshfly filaments, this is mainly due to the loss of myosin in the buffer with glycerol (fig. 3, lane G/ff). Myosin bands are barely visible on SDS gels if relaxing solutions containing formamide are used (fig. 3, left side). In this case, mainly regulatory proteins and actin (bee and locust) seem to be lost. The fleshfly filaments again seem to be special. The pattern of the formamide supernatant (fig. 3, lane F/ff) does not show any actin but major bands of proteins with apparent molecular weights between 55 kD and 80 kD. They might be partly due to arthrin (55 kD) and troponin I (86 kD, see Bullard et al.[2]).

Gradients built of formamide, therefore, were used to separate thin and thick filaments of different insect muscles. Myosin was present in each of the 8 fractions of the density gradient. This might be due to the breakage of the myosin filaments into different-sized fragments by sonication of myofibrils. Only the first two fractions (at the bottom of the tube) contained little actin (fig. 3) but many fragments of thick filaments of various lengths (fig. 1 b). Fig. 3 shows the protein patterns of these fractions (proteins ≤ 210 kD). 7.5% gels are not suited for the separation of proteins with molecular weights > 210 kD[1, 6, 7] or < 36 kD. These proteins, like myosin light chains (17 kD and 30 kD)[1] or projectin (> 600 kD)[6] could be observed on gradient gels (2–15%) of thick filament preparations (not shown). Protein bands corresponding to molecular weights of 105 kD (presumably paramyosin; very faint in fleshfly preparations), 130 kD to 135 kD, and 4 bands from 160 kD to 190 kD, are visible in the patterns of myofibrils and of isolated thick filaments of all investigated muscles. Locust filaments show an additional band at 82 kD, and the filaments of any of the asynchronous muscles, bands at 65 kD (weak in bee). It is not yet clear which of the bands represent genuine proteins, nor whether the complete protein pattern of the thick filaments is visible on the gels.

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