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Purification of Insect Myosin and α-Actinin

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A method is described for preparing insect myosin, tropomyosin and α-actinin. The amino acid compositions of the myosin and α-actinin are given, and some of the properties of the purified proteins are discussed.

Insect fibrillar flight muscle possesses several unique characteristics that make it especially suitable for study of muscle contraction (Pringle, 1967*a*,*b*). Although ultrastructural (Reedy *et al.*, 1965; Reedy, 1968), X-ray diffraction (Tregear & Miller, 1969; Armitage *et al.*, 1973) and mechanical (White, 1970; White & Thorson, 1972) studies of insect fibrillar muscle have yielded valuable insights into the mechanism of muscle contraction, the small amount of fibrillar muscle normally available has made biochemical studies of the contractile proteins difficult. We describe here a method for preparing myosin, tropomyosin and α-actinin from a single sample of insect fibrillar muscle. Since Bullard *et al.* (1973*b*) have described the properties of tropomysin from insect fibrillar muscle, only the properties of purified myosin and α-actinin will be discussed here.

Materials and methods

All procedures were performed at 4°C, and glass-distilled deionized water was used throughout. Myofibrils were prepared as described previously (Bullard *et al.*, 1973*b*) from the fibrillar flight muscle of the water bug *Lethocerus cordofanus* or the dung beetle *Heliocopris japetus*. Myosin was extracted by suspending the washed myofibrils obtained from 5–20g of insect muscle in 3vol. (v/w) of 0.1M-sodium pyrophosphate–0.01M-$MgCl_2$, pH7.0. This suspension was stirred gently for 3h and then centrifuged for 70min at 15000*g*. The sedimented material was resuspended in the same solvent, and the pyrophosphate extraction repeated twice for 70min each time and a third time for 13–15h (overnight). All four extracts were pooled, and the residue was kept for extraction of α-actinin and tropomyosin. The combined extracts were clarified at 15000*g* for 1h, filtered through glass wool to remove lipid, and then salted out at 0°C between 0 and 35% and between 35 and 45% or 35 and 60% $(NH_4)_2SO_4$ saturation. Solid $(NH_4)_2SO_4$ was added slowly to the extract, which was being mechanically stirred. The pH of the extract was maintained between 6.5 and 7.0 by adding 4.0M-KOH as needed; paramyosin was precipitated in the 0–35%-satd.-$(NH_4)_2SO_4$ fraction; if, however, the pH fell below 6.5, myosin was also precipitated. Subsequent work has shown that the narrower range of 35–45%-satd. $(NH_4)_2SO_4$ produces a purer myosin than the 35–60%-satd. range with little loss in yield. Starr & Offer (1971) have previously shown that contaminating proteins are precipitated from rabbit myosin extracts if they are salted out above 45%-satd. $(NH_4)_2SO_4$. After centrifugation at 15000*g* for 20min, the myosin-containing $(NH_4)_2SO_4$ fractions were dissolved in 0.5M-KCl–0.01M-sodium phosphate or in 0.5M-KCl–0.01M-Hepes [2-(*N*-2-hydroxyethylpiperazin-*N*′-yl)ethanesulphonic acid] (pH7.0), dialysed exhaustively against the solvent, and then clarified by centrifuging at 70000*g* for 1h. A small amount of additional purification could be achieved by passing the 35–45%-satd.-$(NH_4)_2SO_4$ myosin fraction through a 4% agarose or a DEAE-cellulose column (Richards *et al.*, 1967).

Tropomyosin and α-actinin could be extracted from the residue after extraction of myosin and paramyosin from the myofibrils. This residue was suspended in 5vol. (v/w, based on original wet weight of muscle) of 1mM-EDTA and treated as previously described (Arakawa *et al.*, 1970; Goll *et al.*, 1970). Since the amount of sample was limited, low-ionic-strength extracts were always salted out between 0 and 30%- and between 30 and 70%-satd. $(NH_4)_2SO_4$ (Robson *et al.*, 1970; Goll *et al.*, 1970, 1972). The 0–30%-satd.-$(NH_4)_2SO_4$ fraction was passed once through a DEAE-cellulose column (Whatman DE-52) as described by Robson *et al.* (1970) to obtain purified α-actinin. The limited amount of sample available made it impractical to attempt additional purification by a second DEAE-cellulose column (Robson *et al.*, 1970), but purity was usually high even after only one column-chromatographic purification (see under 'Results and discussion'). The 30–70%-satd. $(NH_4)_2SO_4$ fraction contained tropomyosin and

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troponin, although the latter protein seemed extensively degraded, possibly from the action of proteolytic enzymes during the long extractions involved in myofibril and myosin preparation. Tropomyosin could be purified by isoelectric precipitation at pH 4.6 in 1 M-KCl followed by salting out with $(NH_4)_2SO_4$ (Bullard *et al.*, 1973*b*).

Amino acid analysis was performed on a Locarte analyser. The proteins were hydrolysed in 5.6 M-HCl at 108°C, and the hydrolysates evaporated at room temperature (22°C) *in vacuo*. Myosin samples were hydrolysed for 24 or 72 h. The values for serine, methionine, threonine and tyrosine were extrapolated to zero time to correct for hydrolytic destruction. The 72 h values were taken for valine and isoleucine; for all others the 24 and 72 h values were averaged. The α-actinin samples were hydrolysed for 24 h only.

Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis was performed by the procedure of Weber & Osborn (1969); 7.5% (w/v) polyacrylamide gels were used throughout. Protein concentrations were measured by the Microbiuret method (Goa, 1953) standardized with Kejdahl N determinations.

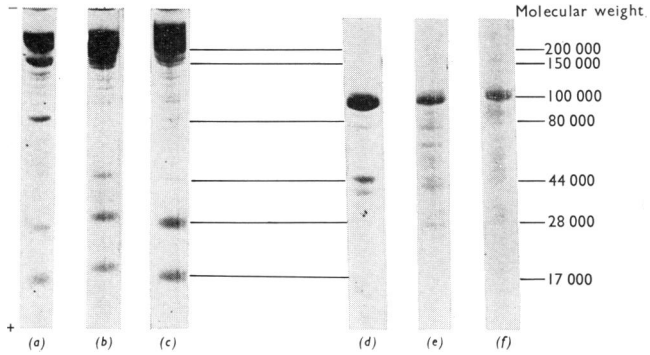
Results and discussion

It was difficult to prepare insect myosin free from paramyosin, since only small quantities of muscle were available. Yet a paramyosin-free myosin is essential for studying the role of paramyosin in thick-filament architecture and the effects of paramyosin or myosin filamentogenesis. Also, a highly purified myosin is necessary for accurate determination of amino acid composition and for antibody studies. Myosin prepared by the procedure described here contained no paramyosin (Plate 1). Although myosin preparations from *Lethocerus* had no major impurities, as judged by polyacrylamide-gel electrophoresis (Plate 1), myosin preparations from the dung beetle contained proteins with subunit molecular weights of 150000 and 80000 (Plate 1*a*). Since the quantity of the 200000-dalton subunit of myosin seemed to decrease as these two impurities increased, and since the subunit molecular weights of heavy and light meromyosin are approx. 150000 and 80000 respectively, we believe that these two contaminants in dung-beetle myosin are heavy and light meromyosin produced by proteolytic digestion of the 200000-dalton subunit during preparation. That the amounts of the 150000- and 80000-dalton subunits relative to the 200000-dalton subunit vary among different preparations suggests that the amount of proteolytic breakdown varies with each preparation. Yields of the purified 35–45%-satd.-$(NH_4)_2SO_4$ fraction were approx. 20 mg/g wet wt. of muscle from the dung beetles and 6–12 mg/g wet wt. of muscle from the water bugs. The yield of myosin obtained when insect muscle was extracted with modified Hasselbach–Schneider solution containing 1 M-KCl, 0.02 M-sodium phosphate buffer (pH 6.5), 1 mM-$MgCl_2$ and 0.01 M-sodium pyrophosphate was much lower (2–2.5 mg/g wet wt. of muscle), and column chromatography or $(NH_4)_2SO_4$ precipitation is necessary to remove paramyosin from these conventional preparations.

ATPase (adenosine triphosphatase) activity of myosin prepared by this new procedure is insensitive to Ca^{2+} concentration because the myosin control system is lost on precipitation (Lehman *et al.*, 1974). Ostensibly, there is no way of removing paramyosin without losing the myosin control system. However, myosin prepared as described here has Ca^{2+}- and actin-activated ATPase activities comparable with or slightly higher than those for desensitized insect myosin prepared as described previously (Bullard *et al.*, 1973*a*). Addition of paramyosin inhibited the actin-activated ATPase of purified myosin prepared as described here. Szent-Gyorgyi *et al.* (1971) have previously observed that paramyosin inhibits actin-activated ATPase in the mollusc system. Tropomyosin has previously been observed to activate the ATPase of actomyosins prepared from insect (Bullard *et al.*, 1972, 1973*a*) or *Limulus* (Lehman & Szent-Gyorgyi, 1972) muscle, and tropomyosin also elicits a twofold increase in the ATPase activity of insect actomyosin made with these preparations of paramyosin-free myosin. Since this activation occurs with actomyosin made from paramyosin-free myosin, it cannot be attributed to removal of the inhibiting effects of paramyosin, but must be a direct effect on the actin–myosin interaction. Indeed, tropomyosin activated paramyosin-free actomyosin ATPase to approximately the same extent as it did the activity of actomyosin made with myosin that was not paramyosin-free.

Amino acid composition of the paramyosin-free insect myosins (Table 1) is similar to but not identical with that previously reported for rabbit (Lowey & Cohen, 1962) and *Phormia* (Kominz *et al.*, 1962) myosins. Insect myosin has more glutamic acid and less lysine than myosin from rabbit muscle.

As judged by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis, insect α-actinin is reasonably well purified by a single chromatographic separation on DEAE-cellulose (Plate 1). The major protein in insect α-actinin preparations has a subunit molecular weight of 95000–100000; this is very similar to the subunit molecular weight reported for mammalian α-actinins (Robson & Zeece, 1973), and indeed *Lethocerus* α-actinin co-migrates with rabbit α-actinin when both are run on the same gel (Plate 1). The amino acid composition of insect α-actinins is also similar to but not identical with that of α-actinin from the rabbit (Robson *et al.*, 1970) or pig (Suzuki *et al.*, 1973). The major difference in amino acid composition between mammalian and insect α-actinins is the slightly higher lysine, tyrosine and



EXPLANATION OF PLATE 1

Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis of insect myosin and α-actinin preparations

(*a*) Dung-beetle myosin, prepared by precipitation between 35 and 45%-satd. $(NH_4)_2SO_4$; 37μg of protein loaded on the gel. (*b*) *Lethocerus* myosin prepared by precipitation between 35 and 45%-satd. $(NH_4)_2SO_4$; 37μg of protein loaded on the gel. (*c*) *Lethocerus* myosin from (*b*) after passage through a column (2.5cm × 88cm) of 4% agarose followed by precipitation between 0 and 60%-satd. $(NH_4)_2SO_4$; 37μg of protein loaded on the gel. (*d*) Dung-beetle α-actinin prepared as described in the text; 37μg of protein loaded. (*e*) *Lethocerus* α-actinin prepared as described in the text; 25μg of protein loaded. (*f*) Rabbit and *Lethocerus* α-actinin run on the same gel. Rabbit α-actinin was prepared as described by Goll *et al.* (1972) and *Lethocerus* α-actinin was prepared as described in the text; 12μg of protein from each species was loaded.

(*Facing p.* 190)

Table 1. *Amino acid composition of insect myosin and α-actinin*

For details see the text.

	Myosin (residues/10^5 g of protein)				α-Actinin (residues/1000 residues)		
Amino acid	Rabbit*	Adult *Phormia*†	Dung beetle	*Lethocerus*	Porcine white‡	Dung beetle	*Lethocerus*
Lys	92	72	78.7	83.9	52.7	65.1	71.2
His	16	13	13.4	15.4	21.6	25.6	22.7
Arg	43	46	49.4	48.3	69.4	66.3	62.8
Asp	85	88	85.8	84.8	105.6	99.3	109.6
Thr	44	30	36.1	38.4	44.9	57.5	57.6
Ser	39	36	39.1	40.0	53.8	52.8	47.6
Glu	157	158	172.8	171.8	163.2	170.2	176.7
Pro	22	19	19.0	24.1	37.9	37.3	43.6
Gly	40	44	47.4	45.4	65.3	60.1	56.7
Ala	78	81	83.1	76.8	94.9	79.1	81.1
Val	43	30	35.9	39.6	40.6	45.4	41.5
Met	23	15	16.6	21.2	28.0	27.0	26.6
Ile	42	32	39.4	34.6	38.5	54.5	47.0
Leu	81	88	93.3	85.1	100.9	99.1	102.2
Thr	20	17	18.0	18.6	22.2	36.2	29.2
Phe	29	24	26.0	26.1	31.8	34.1	35.7
Lys+Arg	135	118	128.1	132.2	122.1	131.4	134.1
Asp+Glu	242	246	258.6	256.6	268.7	269.5	286.3

* From Lowey & Cohen (1962).
† From Kominz *et al.* (1962).
‡ From Suzuki *et al.* (1973); made from the white portion of porcine semitendinosus muscle.

threonine contents in the insect α-actinins. The protein isolated from insect muscle could be identified with α-actinin by its solubility properties, mobility on gels, amino acid composition and elution pattern from DEAE-cellulose columns, although the very small amounts of protein that could be obtained from insect fibrillar muscle (0.5–2 mg from 5–20 g of muscle) precluded its identification as a 6S peak in the analytical ultracentrifuge. Although the very small amounts of muscle and α-actinin available make exact comparison hazardous, it is noteworthy that insect muscle, which has wider Z-discs than mammalian muscle, also yields about twice as much α-actinin per g of muscle than mammalian muscle (cf. Robson *et al.*, 1970).

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