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New proline-rich proteins in isolated insect Z-discs

Gillian M. SAINSBURY* and Belinda BULLARD†
ARC Unit of Muscle Mechanisms and Insect Physiology, Department of Zoology, South Parks Road, Oxford OX1 3PS, U.K.

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Z-discs were isolated from *Lethocerus* (waterbug) flight muscle by removing the contractile proteins from myofibrils with a solution of high ionic strength. Sodium dodecyl sulphate (SDS)/polyacrylamide-gel electrophoresis confirmed a previous report that major Z-disc proteins had subunit mol.wts. of 200000, 180000, 105000, 95000, 42000 and 35000. A protein of subunit mol.wt. 25000 was found in once-washed Z-discs but was degraded or was removed by successive washes. In addition, a protein of high molecular weight (>300000) was found in Z-discs. Proteins of subunit mol.wts. 42000, 35000 and 25000 were individually sliced from SDS/polyacrylamide gels and eluted. Amino acid analysis showed that the 35000-subunit-mol.wt. protein was not, as was previously suggested, tropomyosin, but was a distinct Z-disc protein rich in proline. Calculations based on the amino acid analysis showed that this protein contained substantial hydrophobic regions. Preliminary investigations into the isoelectric point and a method of isolation of the 35000-subunit-mol.wt. Z-disc protein are described. This protein was found in slices cut from SDS/polyacrylamide-gel electrophoretograms of whole myofibrils. The protein of 42000 subunit mol.wt. was shown by amino acid analysis to be actin and the 25000-subunit-mol.wt. Z-disc protein was proline-rich.

The protein composition of the Z-disc has not been fully elucidated, chiefly because of the lack of a pure isolated Z-disc preparation. *Lethocerus* (waterbug) flight muscle has proved to be a suitable tissue for isolation of Z-discs that maintain their structure *in vivo* (Sainsbury & Hulmes, 1977). α-Actinin has been established as a Z-disc protein in many types of muscle by such techniques as antibody staining (Masaki *et al.*, 1967; Schollmeyer *et al.*, 1973), but this protein is thought to constitute only about 17% of the total Z-disc protein (Robson *et al.*, 1970), and α-actinin gives only partial Z-disc reconstruction (Stromer *et al.*, 1969). The other proteins of the Z-disc are not as yet fully characterized, and there is considerable variation in reports of Z-disc components. This may be due in part to methods of Z-disc isolation and in part to species differences. The present paper is concerned with establishing the identity of two Z-disc proteins other than α-actinin. This work arose from the fact that the amino acid analysis of isolated honey-bee (*Apis mellifera*) Z-discs showed that they contained a large proportion of proline (Saide & Ullrick, 1974). This was surprising, since, from SDS/polyacrylamide-gel electrophoresis, the major proteins of insect Z-discs were of subunit mol.wt. 200000 and 105000 (thought to be myosin and paramyosin respectively), α-actinin, actin and tropomyosin, and these proteins could not account for a high Z-disc proline content (Bullard & Sainsbury, 1977). This suggested that there were one or more unidentified proteins with very high proline contents that may have been misidentified from the SDS/polyacrylamide-gel electrophoresis on the basis of subunit weight alone. Examination of the evidence for and against the presence of tropomyosin (subunit mol.wt. 35000) in Z-discs shows that a high-proline-content protein could be mistaken for tropomyosin.

Evidence for the presence of tropomyosin in Z-discs is as follows. First, similarities were noted in the appearance of tropomyosin crystals and cross-sections through vertebrate Z-discs (Huxley, 1963). Secondly, low-ionic-strength extractions of

Abbreviation used: SDS, sodium dodecyl sulphate.

* Present address: University Laboratory of Physiology, Parks Road, Oxford OX1 3PT, U.K.

† Present address and address for reprints: ARC Institute of Animal Physiology, Babraham, Cambridge CB2 4AT, U.K.

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myofibrils, which have been shown to remove Z-discs, reportedly lead to release of tropomyosin [Corsi & Perry, 1958 (rabbit skeletal muscle); Rash *et al.*, 1968 (chick striated and cardiac muscle); Stromer *et al.*, 1976 (rabbit skeletal muscle)]. Similarly, the Z-disc residue from high-ionic-strength extractions of chicken skeletal-muscle myofibrils was thought to contain tropomyosin (Gard & Lazarides, 1979). Thirdly, extracts of rabbit skeletal-muscle myofibrils treated with Ca^{2+}-activated factor ('CAF') contained a protein of 34000 subunit mol.wt. that was tentatively identified as tropomyosin (Suzuki *et al.*, 1978). Fourthly, tropomyosin was thought to be present in isolated Z-discs from insect flight muscle (Saide & Ullrick, 1974; Bullard & Sainsbury, 1977). It is noteworthy that most of the preceding evidence for tropomyosin as a Z-disc protein is based on identification of tropomyosin by its subunit weight obtained from SDS/polyacrylamide-gel analysis. Other evidence suggests that tropomyosin is not a Z-disc protein. First, careful comparison of symmetries in electron micrographs of cross-sections through tropomyosin crystals and vertebrate Z-discs showed that they did not have identical structures [Caspar *et al.*, 1969; Stromer *et al.*, 1969; Goldstein *et al.*, 1977 (canine cardiac muscle)]. Secondly, antibodies to pure tropomyosin do not bind to Z-discs of skeletal muscle (Pepe, 1966; Schollmeyer *et al.*, 1973). Thirdly, pure tropomyosin did not appear to be necessary for reconstruction of Z-discs (Stromer *et al.*, 1969). Fourthly, the protein of subunit mol.wt. 35000 remains in the Z-discs after extraction with 0.4M-KI (Bullard & Sainsbury, 1977). Tropomyosin is soluble in KI and would be expected to be in the KI extract. However the extract contained no protein of that subunit weight.

The first stage of the present work identifies the subunit molecular weight of the proteins present in isolated Z-discs. Values for the amino acid analysis of whole isolated Z-discs confirmed the high proline content of Z-discs. The second stage reports the presence of two hitherto-unidentified proline-rich Z-disc proteins of subunit mol.wt. 35000 and 25000 in insect flight muscle. The present study also throws light on some other Z-disc proteins, such as actin, a protein of 180000 subunit mol.wt. and high-molecular-weight proteins. These are briefly discussed.

A preliminary account of this work was presented at the Eighth European Conference on Muscle and Motility held in Heidelberg, Federal Republic of Germany, in September 1979 (Sainsbury, 1980).

Materials and experimental procedures

Reagents

Standard analytical-grade reagents were obtained commercially. 'Sepramar' reagents for amino acid analysis, PAGE Blue 83 and Nonidet P40 were obtained from BDH, Poole, Dorset BH12 4NN, U.K. DEAE-cellulose DE 52 anion-exchange resin was obtained from Whatman Biochemicals, Maidstone, Kent, U.K. Ampholines were obtained from LKB, South Croydon, Surrey CR2 8YD, U.K.

Experimental procedures

All procedures were carried out at 4°C except where stated.

Preparation of isolated Z-discs. Myofibrils were prepared from the flight muscles of two specimens of the giant waterbug *Lethocerus cordofanus* or one specimen of *Lethocerus maximus* (1–2g muscle) as described previously (Bullard *et al.*, 1973*a*), but with the modification that 0.1mM-phenylmethane-sulphonyl fluoride was added to the sucrose wash medium to inhibit endogenous proteolytic enzyme activity. Myofibrils were inspected with a phase-contrast microscope to ensure that they were relatively free of mitochondria. Z-discs were isolated as described previously (Bullard & Sainsbury, 1977) with the following modifications. Myofibrils were extracted in 1.0M-KCl/0.08M-$NaHCO_3$ buffer, pH 8.9 [myofibrils/buffer, 1:5 (v/v)] at 4°C for 1h. The isolated Z-disc pellet from the high-speed-centrifugation step was washed once in 1.0M-KCl/0.08M-$NaHCO_3$ buffer, pH 8.9, in order to give a high yield of discs.

SDS/polyacrylamide-gel electrophoresis. This was performed essentially by the method of Weber & Osborn (1969) with the following modifications. Gels contained 7·5% (w/v) acrylamide with 0.2% cross-linker. Samples were dialysed against 1% SDS/0.01M-sodium phosphate buffer, pH 7.0, and incubated at 100°C for 3min (except where stated, when samples were incubated at room temperature) in 2% SDS/0.02M-sodium phosphate buffer (pH 7.0)/20% (v/v) 2-mercaptoethanol/12% (v/v) glycerol/0.1% Bromophenol Blue (1vol. of sample to 1vol. of buffer/mercaptoethanol/Bromophenol Blue incubation mixture). Electrophoresis was performed at room temperature for about 3.5h at 8mA per gel tube. Proteins were stained with PAGE Blue 83.

Protein concentrations were measured by the micro-biuret method (Goa, 1953), standardized by Kjeldahl N determination.

The method of elution of individual proteins from SDS/polyacrylamide gels was essentially as described by Stephens (1975), but with some modifications that avoided the use of fluorescence (see Bell, 1979). Proteins at concentrations of 6mg/ml (a total of 7.5mg) were treated as described above for SDS/phosphate/polyacryalmide-gel (7.5%) electrophoresis. Each of six gels (10cm × 1cm) was typically loaded with 0.2ml from the same sample, and gels were run at 9mA/tube for 17h at

room temperature (22°C). Gels were removed from the tubes, blotted dry, placed on a glass plate and marked to a depth of 0·3–0·5 cm at 0.5 cm intervals along their length by pricking with an Indian-ink-loaded syringe needle. Gels were again blotted, rolled in foil, placed in solid-CO_2 powder and deep frozen (−20°C) for 1–2h. Gels were removed from the deep freeze, and a thin slice was cut parallel to the length of each gel, incorporating the surface Indian-ink marks and leaving the remainder of the Indian-ink marks on the gel. The gels were returned to the deep freeze while the slices were stained with PAGE Blue (40min), fixed and destained. The gels were removed from the deep freeze. The required protein band was sliced from the gels with a razor blade while the gels were still partially frozen. The slices were stacked into the elution tube and set into fresh SDS/polyacrylamide (7.5%)-gel mixture. The dialysis tube forming the bag over the end of the gel contained 1 ml of 0.1% SDS/0.01 M-sodium phosphate buffer, pH 6.8 (the electrophoresis-tank buffer). Elution of the proteins was for 6–9 h at 20 mA/tube at room temperature.

Determination of isoelectric point of the 35000-dalton protein. Isoelectric focusing was carried out as described by O'Farrell (1975), with the modifications for resolution of basic proteins (O'Farrell *et al.*, 1977). Ampholines of the range pH 7–9 were used.

Solubilization of isolated Z-discs. Attempts were made to bring isolated Z-discs into solution without addition of SDS. Initially 6·0M-urea/50mM-Tris/HCl/1.0mM-dithiothreitol/0.5 mM-EDTA, pH 8.8, was used, but phase-contrast microscopy revealed that the solution contained a considerable amount of particulate matter, which was shown by SDS/polyacrylamide-gel electrophoresis to contain the full range of Z-disc proteins. The only method found for bringing the Z-disc proteins into solution without SDS was to treat them with non-ionic detergent (Nonidet P40, 4%) together with urea (4.0–6.0M) and 50mM-Tris/HCl, pH 8.0–8.8.

Purification of the 35000-dalton Z-disc protein. Preliminary attempts to isolate the 35000-dalton Z-disc protein have given small dilute yields of the protein. Attempts to concentrate the protein with the Minicon A-25 apparatus (Amicon) were not successful because the protein was adsorbed by the membrane, probably because of the hydrophobic nature of the protein. However, a weak single band of the protein was shown on SDS/polyacrylamide-gel electrophoresis after use of the following method, which takes advantage of the slightly basic nature of the 35000-dalton protein. A pellet of isolated Z-discs was suspended in 6.0M-urea/Nonidet P40 (4%)/50mM-Tris/HCl (pH 8.0)/1 mM-dithiothreitol/1.0mM-EGTA (20–25 mg of protein/ml) and was dialysed overnight against the same solution. The Z-disc solution was centrifuged at 20000g for 15min to remove a small amount of white particulate matter. The supernatant (0.8 ml) was added to a centrifuge tube containing the anion-exchange resin DEAE-cellulose DE52 (3.5 ml) previously equilibrated with 6.0M-urea/4% Nonidet P40/50mM-Tris/HCl (pH 8.0)/1 mM-dithiothreitol/1 mM-EGTA (starting buffer), together with 1.0 ml of starting buffer, mixed, and left at room temperature for 15 min. The cellulose with Z-disc solution was then centrifuged at 10000g for 15 min at 7°C. The cellulose was discarded and the supernatant contained small amounts of a 35000-dalton protein. Passing the Z-disc solution through a column of DEAE-cellulose DE52 was not satisfactory, since, on contact with the Z-disc solution, the cellulose became viscous and frothy so blocking the flow of solution through the column.

Amino acid analysis. Amino acid analysis was performed on a Locarte analyser. Duplicate samples of the proteins (about 0.5 mg) in pellet form (for Z-disc preparations) or in SDS/polyacrylamide-gel-electrophoresis tank buffer (for eluted samples) were hydrolysed *in vacuo* in 5.6M-HCl (to give a final sample size of 0.5–0.6 ml) for 24h at 108°C. The hydrolysates were evaporated to dryness at room temperature (22°C) *in vacuo.* Buffer samples from blank acrylamide gels, treated in the same manner as the protein-loaded gels, were analysed for amino acid content (see Kyte, 1971). Analysis showed the presence of traces of serine and glutamate, which could account for between 2 and 4% of the serine and glutamate in the protein samples eluted from the gels. Glycine from the acrylamide was found to account for up to 8% of the total glycine in eluted proteins. Corrections were not made for these minor contaminants.

Results and discussion

Isolated Z-disc preparation

The protein composition of 15 isolated Z-disc preparations was similar to that shown in Fig. 1 where the Z-disc composition seen on SDS/7.5% polyacrylamide gels is compared with that of the whole myofibril and the soluble extract. There were major proteins of subunit mol.wts. 200000, 180000, 105000, 95000, 42000, 35000 and 25000. Analysis of gel scans showed that proteins of 95000 (α-actinin), 42000, 35000 and 25000 mol.wt. were present in the approximate proportions of 1.0:1.5:1.3:0.7 by weight respectively (assuming all proteins bind the same amount of PAGE Blue dye). A minor band was noted of subunit mol.wt. 70000. The proteins of 180000 and 105000 subunit mol.wt. have not been identified, since the 180000-mol.wt. component could not be separated from myosin, nor the 105000-mol.wt. component from α-actinin. The

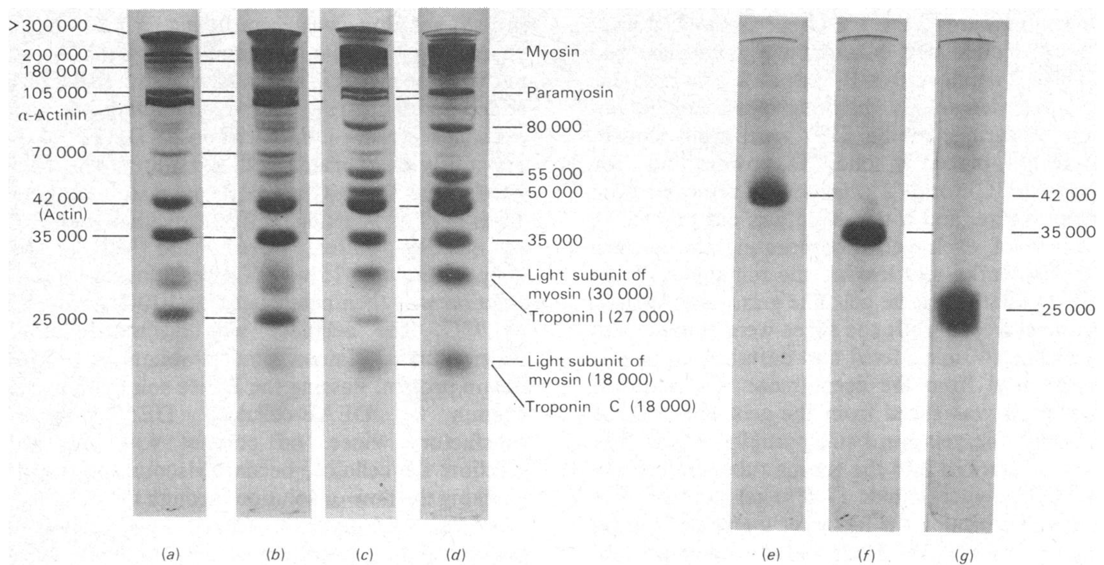

Fig. 1. *Electrophoresis to show the composition of isolated Z-discs from Lethocerus flight muscle, and proteins eluted from gels of isolated Z-discs*

Preparation of samples from *Lethocerus*, the method of SDS/7.5%-polyacrylamide-gel electrophoresis and elution of individual proteins from isolated Z-discs is described in the Materials and experimental procedures section. (*a*) Isolated Z-discs (once-washed); (*b*) isolated Z-discs (once-washed, but containing a small amount of thin- and thick-filament contamination); (*c*) whole myofibrils (note that the protein of 55000 subunit mol.wt. partially degrades to form a band corresponding to 50000 mol.wt.); (*d*) the soluble extract of proteins from whole myofibrils after preparation of isolated Z-discs; (*e*) 42000-subunit-mol.wt. protein (actin) eluted from isolated Z-discs; (*f*) 35000-subunit-mol.wt. protein eluted from Z-discs; (*g*) 25000-subunit-mol.wt. protein eluted from Z-discs.

Z-disc preparation always contained material of a high molecular weight (>300000) that remained on the origin of SDS/5% and 7.5% polyacrylamide gels. It was not possible to elute this material from gels in order to analyse its amino acid composition. Some of this protein may be connectin, which has been located in *Lethocerus* flight muscle and is known to remain on the origin of SDS/7.5% polyacrylamide gels. Connectin has a proline content of 45 residues/1000 (Maruyama *et al.*, 1978). Z-disc proteins of high molecular weight that do not migrate into SDS/polyacrylamide gels have been noted by many workers (see Etlinger & Fischman, 1973; Saide & Ullrick, 1974; Stromer *et al.*, 1976; Suzuki *et al.*, 1978; Gard & Lazarides, 1979).

Two minor differences were noted between the once-washed isolated Z-discs used in the experiments reported in the present paper and those previously described, which were washed two or more times (Bullard & Sainsbury, 1977). First, some preparations contained slightly larger proportions of proteins of subunit mol.wt. 200000 and 42000, and a small band corresponding to 55000 mol.wt., which indicates that, in these, a small amount of thin- and thick-filament contamination was present. (The 55000-subunit-mol.wt. protein partially degrades to form a band corresponding to 50000 mol.wt. on SDS/polyacrylamide gels; see Fig. 1*c*). Figs. 1(*a*) and 1(*b*) show SDS/polyacrylamide-gel electrophoretograms of the range of once-washed Z-disc preparations, most being more like Fig. 1(*a*) than Fig. 1(*b*). Secondly, the once-washed preparation showed a more marked band of protein of subunit mol.wt. 25000 than previously reported (Bullard & Sainsbury, 1977). This protein either becomes degraded or is washed off Z-discs in lengthy washing procedures.

Isolated Z-discs were found to be extremely insoluble in most solutions apart from SDS (see under 'Experimental procedures').

The amino acid analysis of six isolated Z-disc preparations showed small S.D. values for all the amino acids measured (Table 1). This indicates that the Z-disc preparation was reproducible and that the thin- and thick-filament contamination was not significant. The amino acid composition is roughly similar to that found for honey-bee Z-discs by Saide & Ullrick (1974). However, detailed comparison with these authors' results is not practicable because their Z-discs were isolated by a different technique and had a protein composition different from that described here. Table 1 shows that the isolated-Z-disc preparation contained a large proportion of proline (71 residues/1000), which compares with 82

Table 1. *Amino acid analysis of isolated-insect-Z-disc preparations*

Preparation of isolated Z-discs is described in the Materials and experimental procedures section. Values for amino acid of whole isolated Z-discs (column A) are means ± S.D. expressed as residues/1000, and the number of observations is 6. The values in column (B) are mean amino acid contents for the following myofibrillar proteins: *Lethocerus* myosin (Hammond & Goll, 1975); dung-beetle paramyosin (Bullard *et al.*, 1973*b*); *Lethocerus* α-actinin (Hammond & Goll, 1975; *Lethocerus* actin (Bell, 1979); dung-beetle tropomyosin (Bullard *et al.*, 1973*b*).

	Content (residues/1000 residues)	
Amino acid	(A) Isolated Z-discs	(B) Mean for five myofibrillar proteins
Asx	99 ± 8.5	103
Thr	58 ± 5.1	51
Ser	63 ± 5.8	50
Glx	126 ± 8.3	196
Pro	71 ± 5.6	28
Gly	65 ± 4.7	50
Ala	74 ± 3.4	88
Val	51 ± 2.1	51
Met	21 ± 2.5	24
Ile	49 ± 1.3	50
Leu	85 ± 2.8	102
Tyr	42 ± 3.9	24
Phe	37 ± 5.8	26
His	25 ± 0.5	17
Lys	74 ± 2.3	81
Arg	63 ± 1.8	64

residues/1000 in isolated honey-bee Z-discs (Saide & Ullrick, 1974). The mean proline content for myosin, paramyosin, α-actinin, actin and tropomyosin (which, from their subunit weights, were thought to be the Z-disc proteins) was 28 residues/1000, which is far short of the actual Z-disc proline content (Table 1). In addition, the numbers of residues of the aromatic amino acids were higher in whole Z-discs than expected from the mean of the five Z-disc proteins identified by SDS/polyacrylamide-gel electrophoresis. The amino acid analysis suggested that one or more of the Z-disc proteins had very high proline contents. To identify these proteins, bands of individual Z-disc proteins were sliced from SDS/polyacrylamide-gel electrophoretograms of isolated-Z-disc preparations. Individual proteins or pairs of proteins were eluted and analysed for amino acids (see under 'Experimental procedures' for the techniques used). The SDS/polyacrylamide gels of eluted Z-disc proteins of subunit mol.wt. 42000, 35000 and 25000 showed that each of these proteins could be resolved by the elution technique (Figs. 1*e*, 1*f* and 1*g* respectively).

Amino acid analysis of proteins isolated from Z-discs

Comparison of the amino acid analysis of the 35000-subunit-mol.wt. protein eluted from SDS/polyacrylamide gels of isolated Z-discs with that of purified dung-beetle (*Heliocopris*) tropomyosin (Bullard *et al.*, 1973*b*) showed clearly that this protein was not tropomyosin (Table 2). The most striking differences were that the Z-disc protein had a very large amount of proline (10% of the total residues), as well as higher contents of glycine, tyrosine, phenylalanine and histidine, whereas contents of glutamate/glutamine, alanine and lysine were much lower than those found in pure tropomyosin. The amino acid analysis of the 35000-subunit-mol.wt. protein from isolated Z-discs showed a small peak (corresponding to approx. 17 residues/1000) that eluted at the same position as a standard sample of 3-methylhistidine (not shown in Table 2). The Z-disc 35000-subunit-mol.wt. protein was unlike troponin T in its amino acid analysis. For example, troponin T has proline and glutamate/glutamine contents of 47 and 223 residues/1000 respectively [Greaser & Gergely, 1973 (rabbit skeletal muscle)]. The glycine content of the 35000-subunit-mol.wt. protein (73 residues/1000) showed that it was not like that of collagen (approx. 250 residues/1000).

Two parameters of a protein (calculated from the amino acid analysis) $H\varphi_{av.}$ (average hydrophobicity function; Bigelow, 1967) and the discriminant function, z (Barrantes, 1975) gave values for the 35000-subunit mol.wt. Z-disc protein of 5.23kJ (1251cal)/mol and 0.359 respectively. This indicates that the protein has substantial hydrophobic regions, since values for $H\varphi_{av.}$ and z greater than 4.18kJ (1000cal)/mol and 0.317 respectively are diagnostic of intrinsic membrane proteins.

The total number of basic residues in the 35000-subunit-mol.wt. Z-disc protein (arginine, lysine and histidine) was 175 and of acidic residues (aspartic acid/asparagine and glutamic acid/glutamine) was 150/1000 residues. From work on other muscle proteins it can be assumed that approx. 33% of the glutamic acid/glutamine is charged glutamate at physiological pH. Thus the 35000-mol.wt. protein would appear to be positively charged under physiological conditions. The fact that the proline-rich Z-disc protein is basic and has a high proline content indicates that SDS/polyacrylamide-gel electrophoresis may overestimate the subunit weight, as is seen with some histones and a light chain of myosin (see Lowey & Holt, 1973).

In order to see whether the 35000-subunit-mol.wt. Z-disc protein was present in the whole myofibril and to exclude the possibility that it was merely a breakdown product of a higher-subunit-weight protein that appeared during the Z-disc-isolation

Table 2. *Amino acid analysis of some insect Z-disc proteins compared with pure proteins of similar subunit weights from insect myofibrils*

The method for elution of individual or pairs of proteins from isolated Z-discs is described in the Materials and experimental procedures section. Values are means ± S.D. for the number of observations (n) given in parentheses.

	Content (residues/1000 residues)						
Amino acid	Z-disc 42000-subunit-mol.wt. protein	Actin (subunit mol.wt. 42000)*	Z-disc 35000-subunit-mol.wt. protein	Tropomyosin (subunit mol.wt. 35000)†	Z-disc 25000-subunit-mol.wt. protein	Z-disc 200000- and 180000-subunit-mol.wt. proteins	Myosin (subunit mol.wt. 200000)‡
Asx	95 ± 16	87	82 ± 6.1	134	104 ± 6.8	96 ± 18	99
Thr	64 ± 3.5	69	51 ± 5.1	32	45 ± 0.6	55 ± 8.3	45
Ser	64 ± 1.5	67	72 ± 5.6	40	57 ± 11.4	79 ± 7.8	47
Glx	105 ± 25	117	68 ± 8.3	250	96 ± 8.0	140 ± 16.8	201
Pro	54 ± 3.9	58	103 ± 7.0	4	122 ± 5.9	47 ± 9.6	28
Gly	91 ± 5.0	86	73 ± 8.5	25	64 ± 4.7	101 ± 14.6	53
Ala	83 ± 2.5	85	59 ± 4.4	102	75 ± 4.5	85 ± 10.7	90
Val	60 ± 7.1	58	46 ± 11.5	48	33 ± 4.7	68 ± 12.0	46
Met	29 ± 7.6	36	17 ± 3.1	21	13 ± 7.8	15 ± 6.5	25
Ile	64 ± 7.0	72	56 ± 1.7	27	50 ± 9.3	46 ± 10.3	41
Leu	79 ± 5.9	75	86 ± 1.5	118	83 ± 1.0	83 ± 5.5	100
Tyr	36 ± 8.9	43	66 ± 2.5	10	61 ± 18.4	19 ± 7.2	22
Phe	39 ± 4.6	37	46 ± 4.7	15	39 ± 5.3	35 ± 4.6	31
His	27 ± 7.0	24	44 ± 6.0	4	33 ± 3.2	23 ± 4.2	18
Lys	55 ± 5.1	53	48 ± 5.0	103	56 ± 8.2	79 ± 13.5	98
Arg	55 ± 4.5	48	83 ± 2.5	66	69 ± 5.0	55 ± 4.7	57
	(n = 5)		(n = 4)		(n = 3)	(n = 6)	

* From Bell (1979) (*Lethocerus* actin).
† From Bullard *et al.* (1973*b*) (dung-beetle tropomyosin).
‡ From Hammond & Goll (1975) (*Lethocerus* myosin).

procedure, the band corresponding to subunit mol.wt. 35000 was cut from SDS/polyacrylamide gels of whole myofibrils. The composition of this band was intermediate between that of tropomyosin and the Z-disc protein (results not shown). This shows that the Z-disc protein was present in myofibrils in addition to tropomyosin, and confirms that there is a hitherto unidentified protein of subunit mol.wt. 35000 in Z-discs.

The amino acid composition of the 25000-subunit-mol.wt. protein eluted from gel electrophoretograms of isolated Z-discs indicated that this protein was also rich in proline (12% of the total residues) (Table 2). It contained a small, rather variable, amount of amino acid eluting at the 3-methylhistidine position (not shown in Table 2). When eluted from isolated Z-discs, the 25000 mol.wt. protein ran as rather a wide band on SDS/polyacrylamide gels (Fig. 1*g*). This suggests that it may degrade slightly during the elution procedure. Calculation of the parameters $H\varphi_{av.}$ and z for this protein gave values of 5.12kJ (1226 cal)/mol and 0.293 respectively. This showed that the protein was less hydrophobic than the 35000-subunit-mol.wt. protein. This protein was calculated to be basic at physiological pH but carried less positive charge than the 35000-subunit-mol.wt. protein. The amino acid analysis of the 25000-subunit-mol.wt. protein shows that it is not troponin I (subunit mol.wt. 27000) (see Greaser & Gergely, 1973).

The amino acid analysis of the 42000-subunit-mol.wt. protein confirmed that it had been correctly identified by previous workers as actin and this eliminated it as a major proline source. Two of the five samples for amino acid analysis of this protein showed low glutamic acid/glutamine contents (89 and 85 residues/1000). This gave a low mean value and a large S.D. for these amino acids. There is no obvious explanation for this. Some of the actin in the once-washed isolated-Z-disc preparation is contamination from attached thin filaments (see Gard & Lazarides, 1979); however, even after exhaustive extraction of isolated Z-discs and several washes (which remove almost all the thin filaments), the ratio of actin to the 35000-subunit-mol.wt. protein was approx. 1:1.

The pair of bands corresponding to subunit mol.wts. 200000 and 180000 was cut from SDS/polyacrylamide-gel electrophoretograms of isolated Z-discs and analysed together for amino acid composition (Table 2). These proved to be a minor proline source. The eluted proteins showed considerable variation in amino acid composition

between experiments. This was probably due in part to difficulty in selecting the correct bands for slicing from the frozen gels and partly because of the difference in amounts of myosin contamination in once-washed Z-disc preparations. However, assuming that the two bands were approx. 1:1 in ratio and one was myosin (subunit mol.wt. 200000), then the band corresponding to 180000 subunit mol.wt. had lower glutamate/glutamine, leucine, lysine and methionine, and higher serine, proline, valine and glycine contents than has myosin. The component of 180000 mol.wt. may be that of connecting protein linking myosin filaments to the Z-disc (Bullard *et al.*, 1977). It was previously suggested that the protein of subunit mol.wt. 105000 was paramyosin (Bullard & Sainsbury, 1977). However, this is unlikely, because labelling the myofibril with specific antibody suggests that paramyosin is not present at the ends of the thick filaments or on the Z-disc (Bullard *et al.*, 1977). The protein of 105000 subunit mol.wt. may be a component of connecting protein (B. Bullard, unpublished work).

Isoelectric point of the 35000-subunit-mol.wt. Z-disc protein

Preliminary investigations indicated that the isoelectric point of the 35000-subunit-mol.wt. Z-disc protein was approx. pH 7.6. However, the non-equilibrium-pH-gradient-electrophoresis method for basic proteins cannot give accurate values for isoelectric points of proteins (O'Farrell *et al.*, 1977).

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