

MYOFIBRILLAR PROTEIN COMPOSITION CORRELATES WITH HISTOCHEMISTRY IN FIBRES OF THE ABDOMINAL FLEXOR MUSCLES OF THE NORWAY LOBSTER *NEPHROPS NORVEGICUS*

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Summary

The myofibrillar proteins in fibres from the abdominal flexor muscles of the Norway lobster, *Nephrops norvegicus*, have been identified using SDS–PAGE gel electrophoresis. Several contractile and regulatory proteins are expressed as multiple isoforms in single fibres and, according to these, one fast fibre phenotype (F) can be identified in the deep flexor muscles and two slow fibre phenotypes (S₁ and S₂) can be distinguished in the superficial flexor muscles. The two slow fibre phenotypes are distributed non-uniformly across the superficial flexor muscle, and in the lateral bundle there is a heterogeneous mixture of both S₁ and S₂ fibres. Using histochemical procedures applied to intact or freeze-dried fibres in conjunction with measurements of fibre sarcomere length and gel electrophoresis, an exact correspondence can be demonstrated between the morphological properties, enzymatic content and myofibrillar protein composition of individual fibres from the deep and superficial flexor muscles. In the superficial flexor muscle, fibres of the S₁ phenotype have a mean sarcomere length of <8 µm, a low oxidative capacity and an acid-labile isoform of myosin ATPase, while fibres of the S₂ phenotypes have a longer sarcomere length (mean >9 µm), a higher oxidative capacity and an acid-stable isoform of myosin ATPase. These results are discussed in terms of the relationships between the different muscle fibre properties and the usefulness of procedures applied to single fibres for determining them.

Introduction

Crustacean muscle fibres can be divided into two main categories, fast and slow, on the basis of a number of morphological, physiological and histochemical criteria (Govind and Atwood, 1982; Silverman *et al.* 1987).

The most extensively used histochemical methods for discriminating fast and slow crustacean muscle fibres involve tests both for oxidative capacity, indicative of fatigue resistance, and of myofibrillar ATPase activity, indicative of contractile speed. These two

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properties tend to vary inversely, slow fibres having a higher oxidative capacity and lower myofibrillar ATPase activity than fast fibres (Ogonowski and Lang, 1979). A derivative of the ATPase test, which involves a preincubation stage at acid or alkali pH, reliably distinguishes two populations of fibres which contain either a labile or a stable isoform of myosin ATPase (Silverman and Charlton, 1980; Tse *et al.* 1983; Fowler, 1990). In combination with the other methods, this pH-sensitive test can act as a convenient discriminator of fibre types. A number of such studies suggest that two subpopulations of fibres exist in crustacean slow muscles (Kent and Govind, 1981; Govind *et al.* 1981, 1986; Costello and Govind, 1983; Rossi-Durand and Pagni, 1986; Fowler and Neil, 1992) and that up to three subpopulations of fast fibres exist in certain leg muscles (Maier *et al.* 1984, 1986).

Crustacean muscle fibres can also be divided into a number of phenotypic categories according to their myofibrillar protein assemblages, as determined by gel electrophoresis (Lehman and Szent Györgyi, 1975; Silverman *et al.* 1987). This technique has demonstrated that multiple isoforms of certain contractile and regulatory proteins are expressed in single fibres (Costello and Govind, 1984; Quigley and Mellon, 1984; Mykles, 1985*a*). Reference to these can provide an unambiguous identification of fibre phenotypes (Mykles, 1985*b*; Govind *et al.* 1986), and in this way one fast phenotype and two slow fibre phenotypes have been distinguished in different regions of lobster and crab claw muscles (Mykles, 1985*b*, 1988).

By combining this approach with the histochemical analysis of fibre types in the different regions of fast and slow muscles in crab claws, Mykles (1988) has demonstrated that a relationship exists between the histochemical properties of fibres and their myofibrillar protein composition. Even within slow muscles, fibre phenotypes S₁ and S₂ can be equated to fibres with higher and lower levels of myofibrillar ATPase respectively. How widespread and consistent is this relationship in different crustacean muscles, particularly those that display a mixture of histochemical fibre types? An appropriate system in which to address this question is the abdominal postural muscle system of the Norway lobster, since these slow muscles are heterogeneous in composition according to a number of histochemical criteria (Fowler and Neil, 1992). Although Mykles (1985*b*, 1988) has reported results obtained from the electrophoresis of these abdominal muscles in the lobster *Homarus americanus*, the samples analysed were pooled from several fibres and so cannot be compared with the histochemical results obtained from individual fibres.

Therefore, in this study we have performed both a histochemical analysis and a comprehensive survey of the myofibrillar protein composition of fibres in the heterogeneous abdominal superficial flexor (SF) muscles of *N. norvegicus*. We have also developed techniques, based on skinned fibre preparations, for correlating the results of histochemical and biochemical tests and morphological measurements at the level of individual identified fibres. Our results demonstrate that the heterogeneity of the slow muscles is expressed not only in their fibre histochemistry but also in terms of their fibre myofibrillar protein composition. Fibres with a given enzyme composition have a particular myofibrillar protein profile and also a characteristic sarcomere length.

Materials and methods

Animals

Adult male *Nephrops norvegicus* 50–90mm in carapace length were obtained from the Universities Marine Biological Station, Millport, Isle of Cumbrae, Scotland. They were maintained in communal aquarium tanks with filtered, circulated sea water.

After separating the abdomen into dorsal and ventral parts by longitudinal cuts at the level of the hindgut, the superficial flexor muscles were exposed by removal of the overlying deep muscles. Individual muscles were removed intact at their insertions, together with small portions of the associated cuticle or membrane.

Histochemistry

Whole abdomens, or dissected muscles held at resting lengths, were mounted on a cork disk in OCT mounting medium (Miles Scientific, Naperville, Illinois, USA) and frozen in liquid nitrogen. After equilibration to -20°C , the block was trimmed and serial sections were cut at $20\ \mu\text{m}$ on a cryostat (Bright Starlet 2210). Sections were lifted onto coverslips and air-dried at room temperature.

The oxidative capacity of fibres, indicative of their fatigue resistance, was determined from their succinic dehydrogenase (SDH) activity (Lojda *et al.* 1976). The incubation medium contained 1mol l^{-1} sodium succinate, 0.1mol l^{-1} sodium phosphate and 1.0mg ml^{-1} nitroblue tetrazolium (Sigma N6876). The cryosections were placed in a Petri dish on moist filter paper and a drop of the incubation medium was applied to each coverslip. Incubation was for up to 2h at 40°C . The sections were then dehydrated in an alcohol series, cleared in Histoclear (National Diagnostics, Manville, New Jersey, USA) and mounted in Histomount.

The total myofibrillar ATPase activity of the muscle fibres was determined using a method derived from those of Mabuchi and Sreter (1980) and Snow *et al.* (1982). The lability of myosin ATPase activity at low and high pH was determined using a method derived from that of Maier *et al.* (1984), as modified by Fowler (1990). Details of these methods are given by Fowler and Neil (1992).

SDS–polyacrylamide gel electrophoresis

Discontinuous SDS–PAGE was performed according to the method of Laemmli (1970). Gels containing a 2.5% acrylamide stacking gel and a 10% or 5% separating gel were prepared from a 30% (w/v) acrylamide and 0.8% (w/v) *N,N'*-methylene bisacrylamide stock solution.

Muscle fibres were prepared by putting pieces of muscle into cold glycerination buffer containing 20mmol l^{-1} Tris-acetate (pH7.5), 50% glycerol, 0.1mol l^{-1} KCl, 1mol l^{-1} EDTA and 0.1% Triton X-100 for 2–3h. Individual fibres were then separated and transferred to $100\ \mu\text{l}$ of SDS sample buffer [62.5mmol l^{-1} Tris–HCl (pH6.8), 12.5% glycerol, 1.25% SDS, 1.25% β -mercaptoethanol]. Samples were immediately boiled for 3 min, then stored at -20°C until required.

Samples and standards of known relative molecular mass (M_r) (Sigma Dalton Mark VII-L) were applied to the wells in the stacking gel. The gels were then mounted in a

chamber containing a reservoir buffer of 0.2mol l^{-1} glycine, 25mmol l^{-1} Tris-HCl and 0.1% SDS, and run with applied currents of up to 40mA per gel.

Gels were fixed and stained in 0.2% (w/v) Coomassie Blue in 45% (v/v) methanol and 10% (v/v) acetic acid for up to 8h, and subsequently destained in a methanol/acetic acid mixture. In some cases, gels were further stained with silver. The relative amounts of protein in different bands were determined using scanning densitometry (Hoefer GS 300).

Peptide mapping was performed on the myosin heavy chains using α -chymotrypsin. After electrophoresis (10% SDS-polyacrylamide), gels were stained with 0.3mol l^{-1} copper chloride, which precipitates Tris/SDS complexes in the gel, leaving the proteins as clear bands (Lee *et al.* 1987). Slices containing the myosin heavy chains from one of the three flexor muscle phenotypes were excised from the gel and washed three times in 0.25mol l^{-1} Tris and 0.25mol l^{-1} EDTA at pH9.0, then rinsed in water for 10min. The slices were incubated separately in α -chymotrypsin ($50\mu\text{g ml}^{-1}$ in glycerination solution) for 90min at 20°C . The digestion was stopped by adding 100mmol l^{-1} phenylmethane sulphonyl fluoride (PMSF) in ethanol. Each gel slice was placed directly onto the surface of a 12% SDS-polyacrylamide resolving gel, and electrophoresed at 15mA per gel for 1h.

Procedures on single fibres

Intact fibres

Bundles of intact fibres were clamped at resting length in a caliper device and dissected free from their insertions. Sarcomere lengths of individual fibres in the bundle were determined using optical methods. The bundle was then mounted in a Sylgard dish, and the SDH histochemical test was performed using the drop method. After staining, the fibres were separated according to the intensity of staining at their cut ends and transferred to SDS sample buffer for gel electrophoresis.

Freeze-dried fibres

After exposing the muscles *in situ* within the abdomen, the tissue was frozen in liquid nitrogen in order to fix the fibres at resting length and to retain the integrity of their proteins. Subsequently, the preparation was freeze-dried for at least 2 weeks in a stream of evaporating liquid nitrogen, in order to 'skin' the fibre, i.e. to disrupt the sarcolemma. Specimens prepared in this way could be stored for several months without any significant loss of enzyme activity or contractility (Galler and Rathmayer, 1992).

Bundles of freeze-dried fibres were rehydrated in a glycerol solution, and in some cases they were separated from each other on a microscope slide. Histochemical tests were then applied to the fibres, or fibre bundles, using standard procedures.

After the histochemical test, fibre bundles were separated by microdissection, and the sarcomere lengths (SL) of individual fibres were measured by laser diffractometry (Pollack, 1990). The fibres were then electrophoresed. As a control, the sarcomere lengths of some fibres (or myofibrillar bundles split from fibres) were determined before the histochemical tests, and in some cases these tests were omitted prior to gel

electrophoresis. There was no indication that the histochemical tests interfered with the subsequent electrophoretic procedures.

Results

Morphology of muscles

The abdominal muscles of *N. norvegicus* form two groups, the extensors and flexors, each of which is subdivided into distinct deep and superficial sets. The superficial flexor (SF) muscles form thin sheets along the ventral surface of each abdominal segment and are overlaid by the large masses of the deep flexor (DF) muscles. Each SF muscle is divided into a medial bundle, which contains approximately 20 fibres in a single layer over much of its width, and a lateral bundle, which contains approximately 60 fibres in a layer 3–4 fibres thick.

Myofibrillar protein patterns in fibres of the abdominal flexor muscles

In SDS–PAGE gels, the myosin heavy chain, α -actinin, actin and tropomyosin from fibres of the abdominal DF and SF muscles of *N. norvegicus* migrate as single bands with relative molecular masses (M_r) of 200×10^3 , 95×10^3 , 43×10^3 and 38×10^3 respectively (Fig. 1A). However, numerous variants of other myofibrillar proteins, such as paramyosin, troponin T, troponin I, troponin C and myosin light chains are expressed in these muscles, often as multiple isoforms. These variants correspond closely to those described in a number of different crustacean muscles (Lehman and Szent Györgyi, 1975; Costello and Govind, 1984; Mykles, 1985*a,b*; Nishita and Ojima, 1990, Garone *et al.* 1991; Miegel *et al.* 1992).

In individual fibres of the DF and SF muscles, the variants of certain of these myofibrillar proteins occur in combinations that define three phenotypes, one fast and two slow. All flexor muscle fibres fall into one of these three categories, with no apparent intermediate forms. Fast fibres of DF can be distinguished from slow fibres of SF by the presence of the P₁ isoform of paramyosin (M_r 110×10^3) and a 75×10^3 M_r protein (Fig. 1A). These differences are also obvious in 5% gels (Fig. 1B), and can be quantified using scanning densitometry (Fig. 2).

The distribution of isoforms of other regulatory proteins, most notably troponin T, distinguishes two slow fibre phenotypes, designated S₁ and S₂ by Mykles (1985*b*, 1988). The troponin T₁ isoform (M_r 55×10^3) is absent in some fibres, identifying them as S₁, but is present in others, identifying them as S₂ (Fig. 1A). Two other troponin T variants, T₂ (M_r 49×10^3) and T₃ (M_r 47×10^3) occur in different amounts in all three phenotypes, with T₃ being dominant in the fast fibres (Fig. 2).

The three fibre phenotypes also differ in their assemblages of the smaller regulatory proteins, most conspicuously troponin I. The major isoforms are troponin I₁ (M_r 31×10^3) and I₅ (M_r 25×10^3) in fast fibres, I₁ and I₄ (M_r 26×10^3) in S₁ slow fibres, and I₁, I₂ (M_r 29×10^3) and I₃ (M_r 27×10^3) in S₂ slow fibres (Fig. 1A).

Differences in the troponin C isoforms are more difficult to resolve in SDS–PAGE gels stained with Coomassie Blue. However, from overloaded gels it is possible to discern that

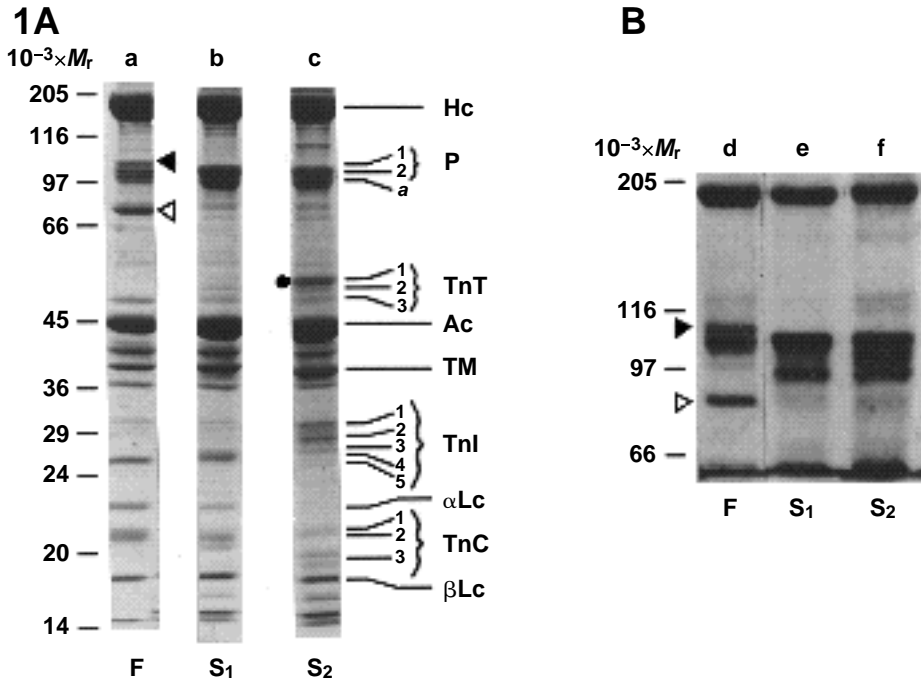


Fig. 1. SDS-PAGE of glycerinated single fibres from the abdominal flexor muscles of *Nephrops norvegicus*. (A) 10% gel stained with Coomassie Blue. (B) 5% gel stained with silver. Ac, actin; *a*, α -actinin; Hc, myosin heavy chain; α Lc, β Lc, myosin light chains; P, paramyosin (two isoforms); TM, tropomyosin; TnC, troponin C (three isoforms); TnI, troponin I (five isoforms); TnT, troponin T (three isoforms). In this and subsequent figures, a filled arrowhead indicates the P₁ isoform of paramyosin, an open arrowhead indicates the $75 \times 10^3 M_r$ protein and a filled circle indicates the T₁ isoform of TnT. The positions of relative molecular mass standards are indicated on the left of the gels. All fibres from the deep flexor (DF) muscles express the F phenotype (lanes a and d), the majority of fibres from the lateral bundle of the superficial flexor (SF) muscle express the S₁ phenotype (lanes b and e) and all fibres from the medial bundle of SF express the S₂ phenotype (lanes c and f).

the F phenotype contains mainly troponin C₂, while the S₁ phenotype has a doublet of the C₂ band, and the S₂ phenotype expresses two unique variants, C₁ and C₃ (Fig. 1A).

Using one-dimensional SDS-PAGE, no differences were apparent in the myosin heavy chains of the different fibre phenotypes. However, these could be distinguished after digestion of the excised heavy myosin bands with α -chymotrypsin, to produce peptide maps. The myosins from F, S₁ and S₂ phenotypes yielded distinctly different populations of digested fragments (Fig. 3), suggesting that they contain different polypeptide sequences. However, the fragments derived from medial and lateral S₂ fibres appeared to be identical.

Distribution of fibre phenotypes in the abdominal SF muscles

The SF muscle is virtually a two-dimensional linear array of fibres. It was therefore possible to remove individual fibres sequentially from one edge of the muscle by

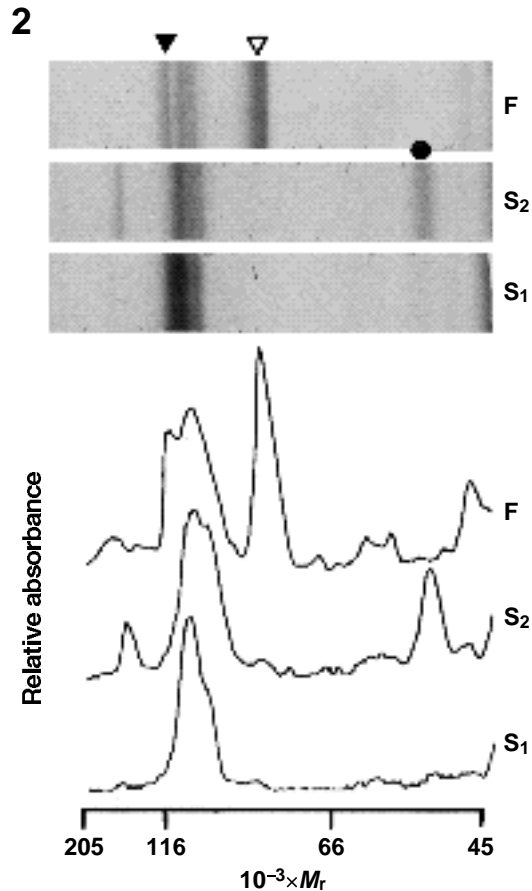


Fig. 2. Scanning densitometry of glycerinated single fibres from the abdominal flexor muscles. Scans of 10% SDS-PAGE gels derived from fibres of the F (top), S₁ (bottom) and S₂ (middle) phenotypes.

microdissection and thus to record their relative positions within the linear fibre array. Following gel electrophoresis, it was then possible to establish the frequency of occurrence of the different fibre phenotypes across the muscle, according to their protein assemblages (Fig. 4A). All fibres in the medial bundle expressed the S₂ phenotype, while in the lateral bundle there was a predominance of fibres of the S₁ phenotype, with a few fibres interspersed which had a phenotypic pattern identical to that of the medial S₂ fibres. In the area of overlap of medial and lateral bundles, there was an approximately equal occurrence of these two phenotypes.

Relationship between electrophoretic properties and muscle histochemistry

In a preliminary survey, the distribution of fibre phenotypes determined electrophoretically was compared with that derived from the histochemical procedures used by Fowler and Neil (1992). Histochemical tests were performed on cryosectioned

SF muscles for the oxidative enzyme SDH, as an indication of fatigue resistance, and for the pH-lability of myosin ATPase after acid preincubation. This combination of tests provides an unambiguous identification of histochemical fibre types (Fowler and Neil,

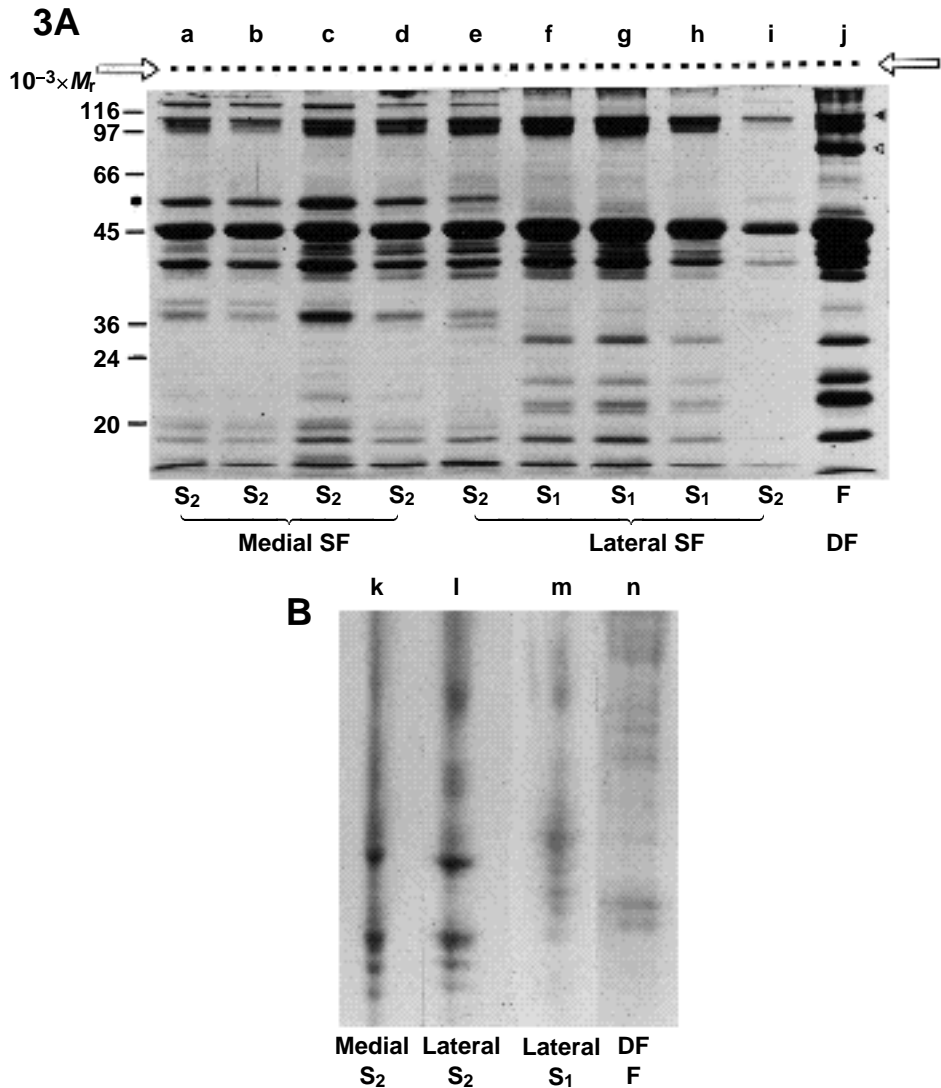


Fig. 3. (A) SDS-PAGE (10% gel) of glycerinated single fibres from the abdominal flexor muscles. Lanes a–d, fibres from the medial bundle of SF (medial SF); lanes e–i, fibres from the lateral bundle of SF (lateral SF); lane j, a fibre from DF. The phenotypic identity of each fibre is indicated below each lane. The dashed line indicates the position of the myosin heavy chains. (B) Peptide map (12% gel) of myosin heavy chains from fibres of different phenotypes in A, obtained by digestion with α -chymotrypsin. Lane k, myosin from lanes a and b (S₂ fibres in the medial SF bundle); lane l, myosin from lanes e and i (S₂ fibres in the lateral SF bundle); lane m, myosin from lanes g and h (S₁ fibres in the lateral SF bundle); lane n, myosin from lane j (F fibre in the DF muscle).

1992). One type, found in both the lateral and medial bundles, has a high level of SDH activity and a pH-stable isoform of myosin ATPase (e.g. fibres a and c in Fig. 5), and the other, found only in the lateral bundle, has a low level of SDH activity and a pH-labile isoform of myosin ATPase (e.g. fibre b in Fig. 5).

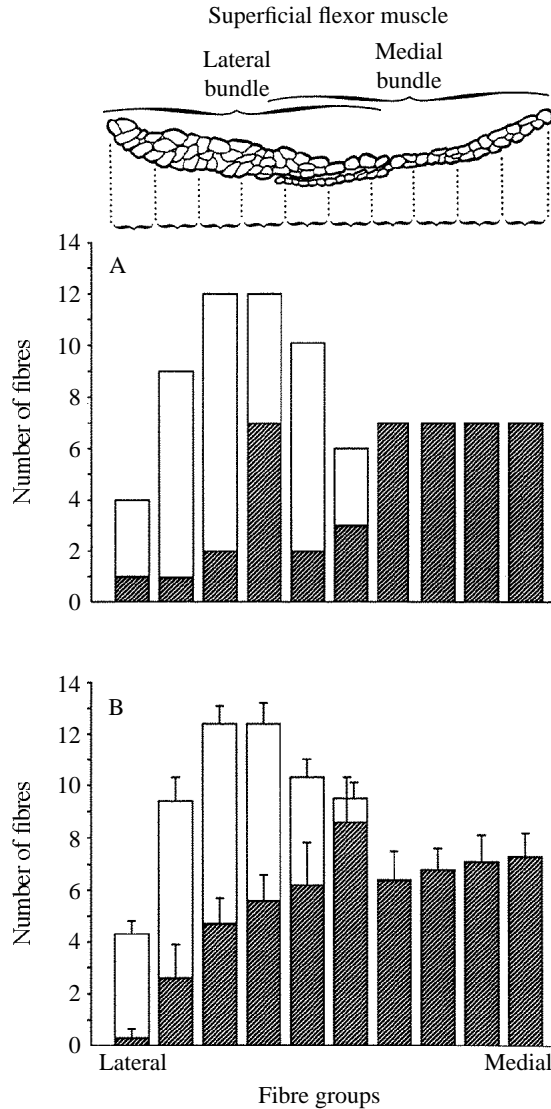


Fig. 4. (A) Distribution of fibre phenotypes across SF, based on SDS-PAGE of individual fibres microdissected from a single muscle. The histogram is constructed from data pooled as shown in the diagram above. Open bars, S₁ phenotype; hatched bars, S₂ phenotype. (B) Distribution of histochemical fibre types across SF, based on the staining reactions of fibres for myosin ATPase following acid preincubation. Data are pooled from muscles of nine individuals. Open bars, labile fibres; hatched bars, stable fibres. Vertical lines indicate one standard deviation.

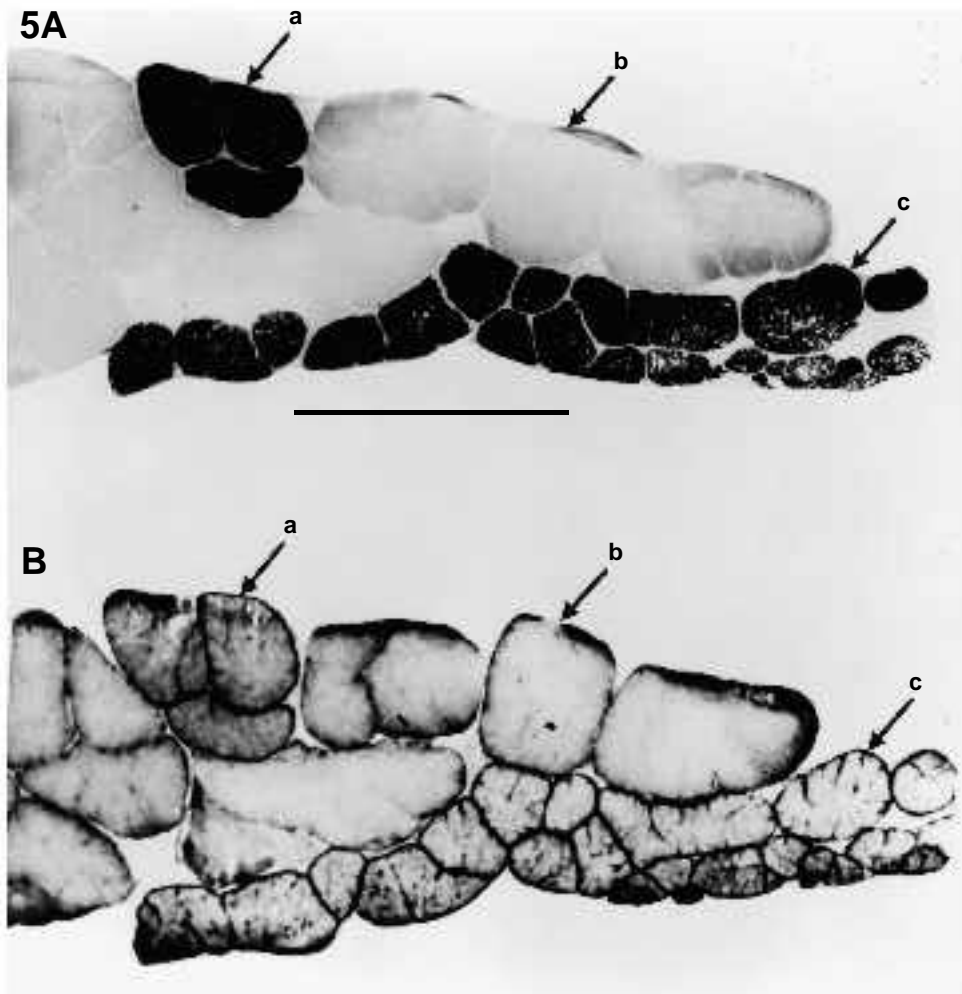


Fig. 5. The histochemical staining properties of a cryosectioned SF muscle. Adjacent sections were subjected to staining for (A) myosin ATPase following acid preincubation and (B) succinic dehydrogenase (SDH). All fibres in the medial bundle (e.g. fibre c) and some fibres in the overlying lateral bundle (e.g. fibre a) are acid-stable and stain strongly for SDH, especially around the subsarcolemmal fringes. The majority of fibres in the lateral bundle (e.g. fibre b) are acid-labile (A) and stain weakly for SDH (B). Scale bar, 0.5mm.

A comparison of histochemical data obtained in this way from the SF muscles of nine animals shows that the distribution of the histochemical fibre types across SF (Fig. 4B) corresponds closely to that of the two phenotypes identified by their myofibrillar protein assemblages (Fig. 4A). Thus, all fibres within the medial bundle express the S_2 phenotype and are histochemically of the high-SDH/stable myosin ATPase type. Within the lateral bundle there is regional heterogeneity of the histochemical fibre types, equivalent to that recorded in terms of their myofibrillar phenotypes. Furthermore, the ratio of the two populations is approximately the same for the two techniques (Fig. 4). This provides

strong indirect evidence that the fibre phenotypes identified from their myofibrillar proteins also differ from each other in their enzymatic properties.

Histochemistry and gel electrophoresis of single intact muscle fibres

In order to correlate myofibrillar protein patterns with muscle histochemistry at the level of individual fibres, we exploited the finding that excised intact fibres can be stained with SDH and discriminated according to the intensity of staining around the peripheries of their cut ends (Fowler and Neil, 1992). The test for myosin ATPase after acid preincubation also yielded results on excised intact fibres, although it produced a variable amount of tissue damage. For this reason, the SDH test was most often performed to indicate a fibre's histochemical type.

The progress of the SDH reaction was followed until differential staining had developed (Fig. 6A). Fibres identified in this way were then microdissected and processed for gel electrophoresis (Fig. 6B). Twenty-five fibres from five muscles were tested with this combined procedure and, in every case, fibres from both the medial and lateral bundles with the higher level of SDH staining had the S₂ phenotypic pattern (16/25), while those from the lateral bundle with the lower level of staining had the S₁ pattern (9/25).

Histochemistry and gel electrophoresis of single freeze-dried muscle fibres

One drawback of the above procedure is that if measurements of sarcomere length are also required it is necessary to hold the intact fibres at resting length in a mechanical device during the dissection procedures. The use of freeze-dried fibres overcomes this problem, since the freezing process effectively fixes the fibres at resting length. However, in order to validate this procedure, it was first necessary to determine whether the histochemical tests and gel electrophoresis yielded the expected results when applied to freeze-dried fibres.

Fibres from the SF and DF muscles which had been skinned by freeze-drying were rehydrated in a glycerol solution, and their sarcomere lengths were determined by laser diffractometry. Measurements were made on 125 fibres taken from 10 separate SF muscle preparations, and 25 fibres taken from five DF muscle preparations. Fibres from the SF medial bundle had long sarcomeres (mean SL $9.6 \pm 1.7 \mu\text{m}$, $N=64$), while fibres from the lateral bundle fell into two populations, one that had similar dimensions to the medial fibres (mean SL $9.4 \pm 1.4 \mu\text{m}$, $N=13$) and another that had shorter sarcomeres ($6.9 \pm 1.2 \mu\text{m}$, $N=48$). Fibres from the DF muscle had sarcomere lengths of less than $4 \mu\text{m}$ (mean $2.8 \pm 0.8 \mu\text{m}$, $N=25$).

When subjected to the SDH histochemical test, freeze-dried fibres showed a staining reaction throughout their whole length (Fig. 7A), in contrast to intact fibres, which reacted only at their cut ends (Fig. 6A). The level of staining differed between fibres, being low in those from DF but more intense in fibres from the SF medial bundle (data not shown). Fibres from the SF lateral bundle reacted in accordance with their sarcomere length measurements: in the example illustrated, those with $\text{SL} > 10 \mu\text{m}$ showed a strong

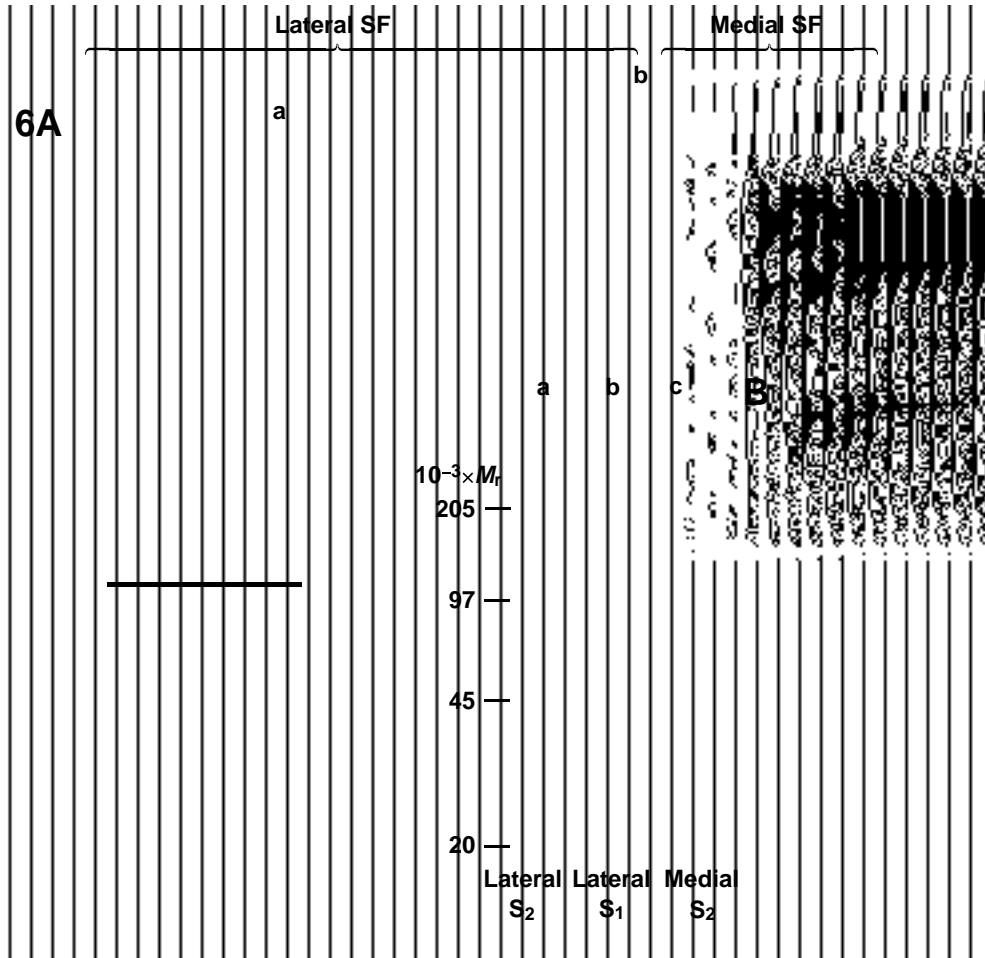


Fig. 6. The correspondence between histochemical and electrophoretic identities of muscle fibres. (A) SDH staining of intact fibres from SF, viewed from their cut ends. The lateral bundle contains both fibres with a high SDH activity (e.g. fibre a) and fibres with a low SDH activity (e.g. fibre b). All fibres of the medial bundle react strongly (e.g. fibre c). Scale bar, 0.5mm. (B) SDS-PAGE 10% gel of the marked fibres in A. The phenotypic identities of the fibres are indicated below the lanes.

reaction (Fig. 7A, top row), while those with $SL < 9 \mu\text{m}$ showed a weak reaction (Fig. 7A, bottom row).

Freeze-dried fibres from the SF and DF muscles were also subjected to gel electrophoresis following histochemical staining. Control tests in which the proteins from freeze-dried fibres were run alongside those from intact fibres confirmed that the skinning process did not alter the pattern of myofibrillar proteins in any significant way; the F, S₁ and S₂ phenotypes remained clearly distinguishable.

Fig. 7B shows the gels obtained from the freeze-dried fibres of the SF lateral bundle following SDH histochemistry (Fig. 7A). The fibres with the longer SL and high levels of

SDH activity expressed the S₂ phenotypic pattern (Fig. 7B, lanes a, c, e, g), while the fibres with the shorter SL and lower levels of SDH activity expressed the S₁ phenotype (Fig. 7B, lanes b, d, f, h). This was a consistent finding in all of the 25 fibres sampled from the lateral bundles of SF that were subjected to this combined protocol.

Discussion

Myofibrillar protein assemblages

Our results on the abdominal muscles of *N. norvegicus* confirm the findings of a number of other studies on crustacean muscle systems (reviewed by Silverman *et al.* 1987) that various contractile and regulatory proteins are expressed as multiple isoforms, even within single fibres. The number of variants of different proteins that are present in our one-dimensional SDS-PAGE gels (two paramyosins, three troponin Ts, five troponin Is and three troponin Cs) suggests that lobster abdominal muscles express a similar range of isoform diversity to that found in the various crab, lobster and snapping shrimp appendage muscles previously studied (Costello and Govind, 1984; Quigley and Mellon, 1984; Mykles, 1985*a,b*, 1988; Govind *et al.* 1986; Nishita and Ojima, 1990; Garone *et al.* 1991; Miegel *et al.* 1992).

Three fibre phenotypes, one fast and two slow, are recognisable according to the presence of a number of key isoforms: paramyosin P₁ and a $75 \times 10^3 M_r$ protein in fast but not slow fibres, and troponin T₁ in the S₂ but not the S₁ slow phenotype. The fact that these differences also characterise the three identified fibre phenotypes in claw muscles (Mykles, 1985*b*) make them useful discriminators of fibre type. They must also reflect important differences in the functional properties of the different fibre types, and Mykles (1988) has suggested ways in which these may be brought about. In order to test these predictions, detailed information is now required on the amino acid sequences and calcium-binding properties of the regulatory proteins in lobster abdominal muscles, using biochemical techniques such as those applied by Kobayashi *et al.* (1989) and Collins *et al.* (1991) to other crustacean muscles.

Relationship between histochemistry and myofibrillar composition

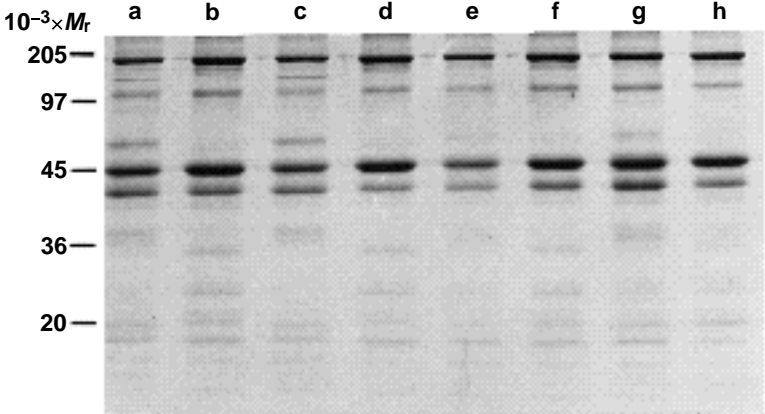
The three fibre phenotypes defined by myofibrillar composition correlate directly with the histochemical fibre types we have determined (Fig. 5A,B and Fowler and Neil, 1992). Furthermore, the combined measurements of sarcomere length, histochemical properties and biochemical identities provide unequivocal evidence that a given histochemical type corresponds to a particular fibre phenotype, with a characteristic myofibrillar protein pattern. The fibre-by-fibre surveys across the SF muscle support this conclusion and confirm that the S₂ phenotype is expressed in an identical form in fibres of both the lateral and medial bundles.

Although not distinguishable in one-dimensional SDS-PAGE runs, peptide mapping reveals differences in the composition of the myosin heavy chains from the fast and the two slow fibre phenotypes. Such differences have also been revealed between fast and slow fibre populations of other crustacean muscles using this and other methods, such as two-dimensional SDS-PAGE (Li and Mykles, 1990). However, the extent to which the

7A



B



Phenotype	S ₂	S ₁	S ₂	S ₁	S ₂	S ₁	S ₂	S ₁
Sarcomere length (μm)	10.9	8.9	10.7	8.3	10.5	8.4	10.5	8.5

Fig. 7. Combined application of sarcomere length measurements, histochemistry and gel electrophoresis to single freeze-dried fibres from the lateral bundle of the SF muscle. (A) SDH staining of rehydrated freeze-dried fibres. Fibres in the upper row (a, c, e, g) are heavily stained throughout their whole length, while fibres in the lower row (b, d, f, h, i, j) are only weakly stained. Scale bar, 1mm. (B) SDS-PAGE 10% gel of fibres stained in A. The sarcomere lengths of the fibres, determined by laser diffractometry before histochemical staining, are indicated below the lanes.

expression of different myosin ATPase isoforms, or different levels of myofibrillar ATPase activity (Fowler and Neil, 1992), are a direct consequence of these differences in myosin composition is yet to be determined.

Muscle heterogeneity

Both the myofibrillar protein patterns and fibre histochemistry discriminate homogeneous and heterogeneous members of the abdominal postural muscle system in *N. norvegicus*. The predominance of heterogeneity in the long-sarcomere fibres of SF is unexpected, since a common interpretation has arisen from morphological and physiological studies that these muscles are uniformly slow and perform a simple postural function in long-bodied decapod crustaceans (Kennedy and Takeda, 1965; Jahromi and Atwood, 1969). Indeed, Mykles (1985*b*, 1988) has reported that the SF of the lobster *Homarus americanus* contains only the S₂ phenotype, and interprets this finding as support for homogeneity in this muscle.

In another study, we have found that the SF muscle of the lobster *Homarus gammarus* displays the same extensive heterogeneity of S₁ and S₂ fibre phenotypes as we report here for *N. norvegicus* (D. M. Neil, W. S. Fowler and G. Tobasnick, unpublished observations). We can only suggest that the failure by Mykles (1985*b*) to find S₁ phenotypes was due to the pooling of fibres from the lateral bundle of the muscle. The nature of the key discriminators is such that a mixture of S₂ and S₁ fibres could certainly be mis-identified as the S₂ phenotype, especially if the troponin C bands were not well resolved. This highlights the need for analyses of fibre phenotypes to be carried out at the level of individual fibres, not pooled samples, in order to detect fibre heterogeneity.

Our finding of regional heterogeneity in the SF muscle of *N. norvegicus* suggests the need for a re-interpretation of its functional role in motor activities. Its two fibre phenotypes have distinct patterns of innervation (Neil and Fowler, 1990), which deliver different levels of synaptic input (Denhean and Neil, 1993). It is now necessary to determine whether these different fibre phenotypes are differentially recruited in different types of motor behaviour and have different contractile properties.

Single-fibre procedures

A number of protocols have been developed in order to correlate the properties of individual muscle fibres. Working on crab leg muscles, Rathmayer and colleagues have combined the cutting and staining of cryosections with a subsequent microdissection of particular fibres from the block for subsequent assay procedures (Maier *et al.* 1986) or mechanical measures (Gunzel *et al.* 1993). We have reversed the order of such procedures, using the SDH test on dissected intact fibres to determine their histochemical

type, and following this by gel electrophoresis. This provides a convenient and reliable method for relating the different properties of a fibre and can be readily extended to include other procedures.

The use of freeze-dried fibres offers further advantages, since it allows the histochemical, biochemical and morphological properties to be directly correlated. It makes fixation at resting length simple to accomplish (so that SL can be measured), preserves enzymatic activity and maintains the integrity of muscle proteins. Moreover, the rehydrated fibres will contract in appropriate calcium solutions, so that it is possible to determine their mechanical properties in relation to their fibre phenotype (S. Galler and D. M. Neil, in preparation). For innervation studies, the additional step of injecting the muscle fibre with dye can be added (Neil and Fowler, 1990).

Thus, the fact that a strict relationship exists between the histochemical properties and myofibrillar profiles of fibres of different phenotypes has great practical utility for investigating their functional properties.

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