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Polymorphism of myofibrillar proteins of rabbit skeletal-muscle fibres

An electrophoretic study of single fibres

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Rabbit predominantly fast-twitch-fibre and predominantly slow-twitch-fibre skeletal muscles of the hind limbs, the psoas, the diaphragm and the masseter muscles were fibre-typed by one-dimensional polyacrylamide-gel electrophoresis of the myofibrillar proteins of chemically skinned single fibres. Investigation of the distribution of fast-twitch-fibre and slow-twitch-fibre isoforms of myosin light chains and the type of myosin heavy chains, based on peptide 'maps' published in Cleveland, Fischer, Kirschner & Laemmli [(1977) *J. Biol. Chem.* **252**, 1102–1106], allowed a classification of muscle fibres into four classes, corresponding to histochemical types I, IIA, IIB and IIC. Type I fibres with a pure slow-twitch-type of myosin were found to be characterized by a unique set of isoforms of troponins I, C and T, in agreement with the immunological data of Dhoot & Perry [(1979) *Nature* (*London*) **278**, 714–718], by predominance of the β-tropomyosin subunit and by the presence of a small amount of an additional tropomyosin subunit, apparently dissimilar from fast-twitch-fibre α-tropomyosin subunit. The myofibrillar composition of type IIB fast-twitch white fibres was the mirror image of that found for slow-twitch fibres in that the fast-twitch-fibre isoforms only of the troponin subunits were present and the α-tropomyosin subunit predominated. Type IIA fast-twitch red fibres showed a troponin subunit composition identical with that of type IIB fast-twitch white fibres. On the other hand, a unique type of myosin heavy chains was found to be associated with type IIA fibres. Furthermore, the myosin light-chain composition of these fibres was invariably characterized by a small amount of LC3F light chain and by a pattern that was either a pure fast-twitch-fibre light-chain pattern or a hybrid LC1F/LC2F/LC3F/LC1Sb light-chain pattern. By these criteria type IIA fibres could be distinguished from type IIC intermediate fibres, which showed coexistence of fast-twitch-fibre and slow-twitch-fibre forms of myosin light chains and of troponin subunits.

Most mammalian skeletal muscles contain at least three types of motor units, namely fast-twitch fast-fatigue, fast-twitch fatigue-resistant and slow-twitch fatigue-resistant units (Burke *et al.*, 1973).

It is now widely accepted that the differences in the pattern of functional activity of muscle fibres are reflected in differences in the ultrastucture (Gauthier, 1971; Eisenberg & Kuda, 1976), energy metabolism (Bass *et al.*, 1969; Spamer & Pette, 1980), protein composition of the myofibrils (Perry, 1974) and protein composition of intracellular membranes (Margreth *et al.*, 1974; Heilmann *et al.*, 1977; Salviati *et al.*, 1982).

The polymorphism of myofibrillar proteins provided criteria for classifying skeletal-muscle fibres, such as those based on the histochemical reaction for myofibrillar (myosin) ATPase, after alkali and acid preincubation (Brooke & Kaiser, 1970), and on differential staining with fluorescent antibodies to the several isoforms of myosin (Sartore *et al.*, 1978; Gauthier & Lowey, 1979) and of the tropomyosin and troponin (Dhoot & Perry, 1979).

A more direct approach to the study of polymorphism is the electrophoretic analysis of the myofibrillar proteins of single fibres. Many recent

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reports have appeared on the electrophoretic pattern of myofibrillar proteins in single fibres from skeletal muscle of the chicken (Mikawa *et al.*, 1981), rabbit (Pette & Schnez, 1977; Julian *et al.*, 1981) and cow (Young & Davey, 1981), as well as from human muscle (Billeter *et al.*, 1981). However, the results obtained in different laboratories are not directly comparable, because of the different experimental conditions for dissecting the muscle fibres, as well as for removing the soluble sarcoplasmic proteins, whose presence can seriously interfere with the identification of some myofibrillar proteins (e.g. tropomyosin subunits; Mikawa *et al.*, 1981; Young & Davey, 1981). Also, the level of detection of proteins in electrophoretic gels of single fibres varies considerably according to the staining method used.

The results reported in the present paper deal with the typing by histochemical criteria and the pattern of composition of myofibrillar proteins, both myosin and regulatory proteins of the I filament, in several skeletal muscles of the rabbit. Most of these muscles had been previously characterized for the light-chain compositions and immunological properties of the isolated myosin (Biral *et al.*, 1982).

In the present work single muscle fibres were prepared by a method previously largely used in physiological and Ca^{2+}-transport activity studies (Wood *et al.*, 1975; Sorenson *et al.*, 1980; Salviati *et al.*, 1982), which involves skinning of the muscle fibres by EGTA treatment and the extraction in glycerol solution. The isoforms of the several myofibrillar proteins were resolved and characterized by one-dimensional polyacrylamide-gel electrophoresis combined with the highly sensitive silver staining method.

Materials and methods

Preparation of chemically skinned fibres

New Zealand White male adult rabbits were used. The animals were killed by stunning and exsanguination. The median portion of psoas muscle (predominantly fast-twitch muscle), the soleus, semitendinosus and crureus muscles (predominantly slow-twitch muscles), and the diaphragm, masseter, gastrocnemius and vastus lateralis muscles (mixed-type muscles) were used. Muscle biopsy samples (bundles 3–5 mm in diameter, 20–30 mm long) were tied to a wooden stick and stretched to 110–120% of slack length before they were cut from the bulk of muscle tissue. Biopsy samples were chemically skinned by incubation at 0–4°C for 24 h in 10 ml of a 'skinning solution' (5 mM-K_2EGTA/170 mM-potassium propionate/2.5 mM-Na_2K_2ATP/2.5 mM-magnesium propionate/10 mM-imidazole buffer, pH 7.0) by the procedure of Wood *et al.* (1975). After 4 h and 8 h the skinning solution was replaced with fresh solution (Salviati *et al.*, 1982). After 24 h the biopsy samples were transferred to a 'storage solution' of the same composition of the skinning solution but also containing 50% (v/v) glycerol, and were stored at −20°C. Control experiments showed that storage for as long as 10 months did not change the electrophoretic pattern of the contractile proteins or their physiological properties (Wood, 1978).

Histochemistry

Single fibres were isolated from muscle bundles under a dissecting microscope, care being taken that the temperature of the glycerol medium did not exceed 10°C. Three segments were cut from the fibre and mounted on microscope glass slides for histochemistry. The remaining segment (usually about 5 mm long) was used for electrophoresis. Staining for myofibrillar ATPase was performed by the method of Padikula & Hermann (1955), with preincubation at pH 10.4, 4.6 and 4.35 (Guth and Samaha, 1969). For correct evaluation of the stain intensity, fibre segments were examined immediately after the reactions were terminated, before drying and mounting (Spamer & Pette, 1977).

One-dimensional electrophoresis

Fibre segments were transferred with a needle to a small capillary tube filled with 20–30 μl of solubilizing solution [10% (v/v) glycerol/5% (v/v) 2-mercaptoethanol/2.3% (w/v) sodium dodecyl sulphate/62.5 mM-Tris/HCl buffer, pH 6.8), and were incubated overnight at room temperature. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed by using a modification of the procedure of Laemmli (1970), with 15% acrylamide or a 10–20% polyacrylamide linear gradient in the separating gel. Gels were cast in vertical slab electrophoresis apparatus, either a Bio-Rad model 220 (100 mm × 140 mm × 0.75 mm; wells 4 mm in width) or an LKB model 2001 (160 mm × 180 mm × 0.5 mm; wells 2 mm in width). The electrophoresis buffer was 0.1% (w/v) sodium dodecyl sulphate/25 mM-Tris/glycine buffer, pH 8.3.

Two-dimensional electrophoresis

Two-dimensional electrophoresis was performed on fibre segments solubilized by incubation overnight at room temperature in 20–30 μl of 9.5 M-urea/2% (v/v) Nonidet NP-40/5% (v/v) 2-mercaptoethanol/1.6% (v/v) Ampholine (LKB) pH 5–7/0.4% (v/v) Ampholine pH 3.5–10 (O'Farrell, 1975). Isoelectrofocusing (first dimension) and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (second dimension) were performed by the method of O'Farrell (1975), as described by Volpe *et al.* (1981). In the second dimension the gel was 0.5 mm thick.

Characterization of proteinase-digest peptide of myosin heavy chains

This was performed by the method of Cleveland *et al.* (1977), with the modifications described by Carraro *et al.* (1981) and Dalla Libera (1981). Myosin heavy chains were separated from other myofibrillar proteins by electrophoresis on 10–20%-linear-gradient polyacrylamide gels, as indicated above. After being stained with Coomassie Blue for 15 min and destained for 15 min, the strip of the gel containing myosin heavy chains was cut out and soaked for 30 min in 20–30 ml of 1 mM-EDTA/1 mM-2-mercaptoethanol/1% (w/v) sodium dodecyl sulphate/125 mM-Tris/HCl buffer, pH 6.8.

Myosin heavy chains were digested with 10 μg of *Staphylococcus aureus* V_8 proteinase (Miles Laboratories) during the run in the stacking gel (about 90 min). In the second electrophoretic system the separating gel (20 cm long) was a 15–22.5%-linear-gradient polyacrylamide gel, and the stacking gel (2 cm long) was a 4.6% polyacrylamide gel. Electrophoresis was performed overnight in a Future Plastic (Boston, MA, U.S.A.) apparatus at a constant current of 12 mA until the voltage output reached 200 V and then at a constant voltage of 200 V. Electrophoresis buffer was 0.25% (w/v) sodium dodecyl sulphate/62.5 mM-Tris/glycine buffer, pH 8.3. After electrophoresis the gel was extensively soaked with 50% (v/v) methanol/12% (v/v) acetic acid and stained with silver as reported below.

Silver staining

Silver staining was performed with the photochemical method of Merril *et al.* (1981). After electrophoresis, gels were fixed in 50% (v/v) methanol/12% (v/v) acetic acid for at least 30 min and then in 10% (v/v) ethanol/5% (v/v) acetic acid for another 30 min, with three changes of the solution. Gels were treated with 3.4 mM-$K_2Cr_2O_7$/3.2 mM-HNO_3 solution for 5 min. After four washes with distilled water the gels were soaked for 30 min under a high-intensity uniform light, at room temperature, in 12 mM-$AgNO_3$ solution. Colour was developed with fresh solution of 0.28 M-Na_2CO_3 containing 0.5 ml of 40% formaldehyde/l, followed by washing with a solution of 1% (v/v) acetic acid. When gels were overstained with silver, the background was cleared by soaking the gels for about 10 min in a solution consisting of 100 ml of distilled water/1 ml of 1% (w/v) $AuCl_3$/0.1 ml of acetic acid. The gels were fixed for 5 min in 5% (w/v) $Na_2S_2O_3$ and stored in distilled water. Densitometric measurements were made as described by Volpe *et al.* (1981).

Coomassie Blue staining

After electrophoresis, gels were stained with 0.25% Coomassie Blue/40% (v/v) methanol/10% (v/v) acetic acid at 60°C for about 2 h and partially destained by several changes of 40% (v/v) methanol/10% (v/v) acetic acid. Finally, protein band stain was intensified by the perchloric acid-staining method of Reisner *et al.* (1975).

Preparation of muscle proteins

Myosin was prepared with the method of Bàràny & Close (1971). Tropomyosin and troponin were purified from rabbit muscles as described by Roy *et al.* (1979).

Determination of protein

Protein was determined with the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Results

General criteria for fibre typing and methodology of staining of electrophoretic gels

Histochemical fibre typing, by myosin ATPase activity, of chemical skinned muscle fibres from soleus and psoas muscles of the rabbit gave results in agreement with the light-chain composition of the constituent myosin. Thus type I slow-twitch fibres all contained myosin light chains LC1Sa, LC1Sb and LC2S, and type II fast-twitch fibres, both IIA (fast-twitch red) (Figs. 6*c*, 6*d* and 6*m*) and IIB (fast-twitch white) fibres (Fig. 1), contained myosin light chains LC1F, LC2F and LC3F exclusively. With regard to myofibrillar proteins other than myosin, the electrophoretic results in the same Fig. 1 are somewhat comparable with those obtained with isolated myofibrils from rabbit skeletal muscle (Etlinger *et al.*, 1976; Porzio & Pearson, 1977), which indicates that chemical fibre skinning is effective in removing soluble sarcoplasmic proteins. The presence of these proteins, such as in ox muscle fibres isolated by different procedure, can seriously interfere with the identification of tropomyosin and troponin components. Under the conditions used, there was found an overall correlation between the distribution of fast-twitch-fibre and slow-twitch-fibre isoforms of regulatory proteins among single muscle fibres and their myosin light-chain composition (Fig. 1), in agreement with previous work (Mikawa *et al.*, 1981). In type I slow-twitch fibres, only the corresponding isoforms of troponins I, C and T were found to be present, as well as a greater amount of β-tropomyosin. These fibres also differed from type II (A and B) fast-twitch fibres by the number of C-protein peptides in the 130000-mol.wt. region. Thus, as in the case of fast-twitch fibres from ox muscle (Young & Davey, 1981), rabbit psoas fast-twitch fibres appear to contain two distinct protein bands in this region, and slow-twitch soleus fibres only one peptide. Further heterogeneity between rabbit fast-twitch and slow-twitch fibres is

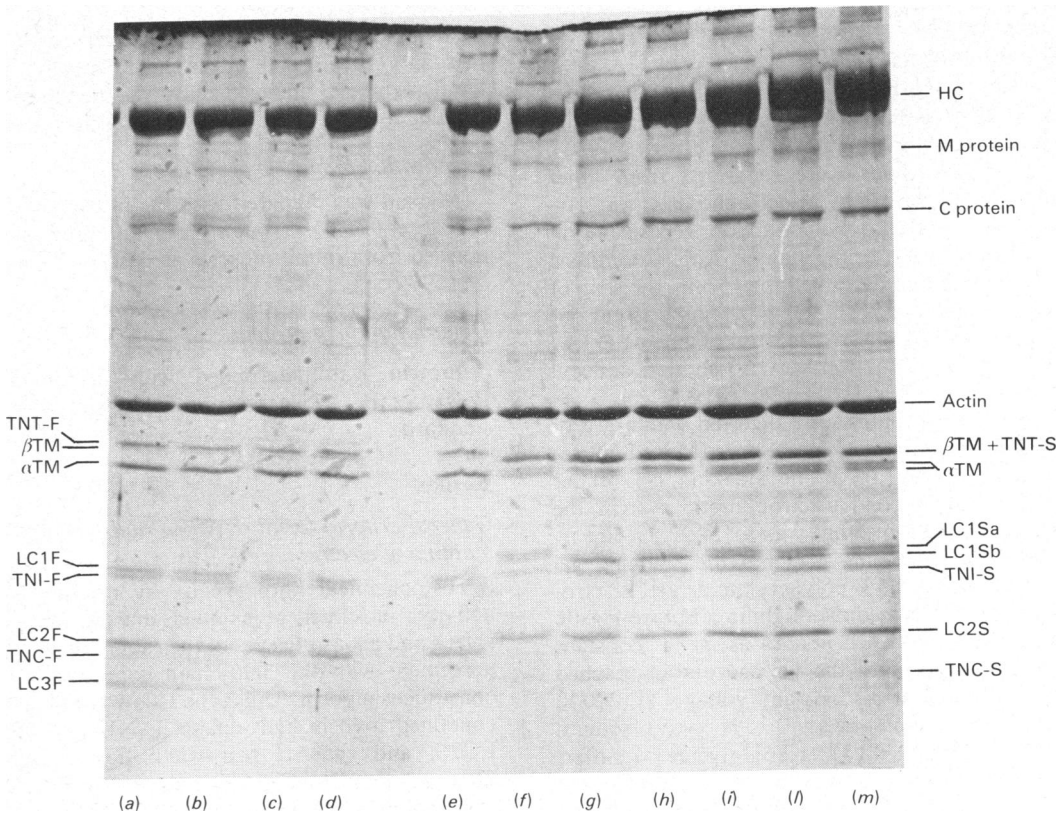

Fig. 1. *Patterns of myofibrillar proteins of chemically skinned type II and type I fibres from rabbit skeletal muscles*
Type II fibres from psoas muscle and types I fibres from soleus muscle were chemically skinned as described in the Materials and methods section. Relatively long fibre segments (20–25 mm) were used. Polyacrylamide-gel electrophoresis was performed by the method of Laemmli (1970) in the LKB apparatus, at a constant current of 10 mA/slab. The separating gel was a 10–20% polyacrylamide linear gradient. After electrophoresis, the gel was stained with Coomassie Blue. (*a*)–(*e*) Type IIB fast-twitch psoas fibres; (*f*)–(*m*) type I slow-twitch soleus fibres. Key: LC, myosin light chains; HC, myosin heavy chains; TM, tropomyosin; TN, troponin. Fast-twitch-fibre isoforms (F) of myofibrillar proteins are indicated on the left side, and slow-twitch-fibre isoforms (S) on the right side.

indicated by the presence respectively of a single or two peptides of molecular weight about 170000, which according to Etlinger *et al.* (1976) are to be identified with M proteins.

Since these results were obtained by detection of the protein bands on electrophoretic gels by staining with Coomassie Blue and since the sensitivity of silver staining was reported to be one to two orders of magnitude higher (Switzer *et al.*, 1979), though with differences according to the particular protein, the two stain methods were compared in our fibre preparations. With purified myosin, it had been established that the minimum amount of protein required for detection of the light-chain components in the electrophoretic gels was about 0.5–1 μg after staining with Coomassie Blue, and as low as 40 ng of protein with the silver staining.

To investigate the differential staining ability of silver towards the several myofibrillar proteins, electrophoretic gels of psoas and soleus fibres, stained with Coomassie Blue (Figs. 2*a* and 2*c*), were destained, and then stained with silver (Figs. 2*b* and 2*d*). It is evident that, after treatment with $AgNO_3$, a marked increase in staining was obtained with myosin light chains and actin. Furthermore, protein bands that were barely visible after Coomassie Blue staining became quite evident after the double staining. One of these bands, migrating between troponin T and actin, was identified with creatine kinase (42000 mol.wt.), according to Etlinger *et al.* (1976). On the other hand, the increase in stain intensity of protein bands after staining with silver was less marked with fast-twitch-fibre and slow-twitch-fibre tropomyosin, as shown by comparison

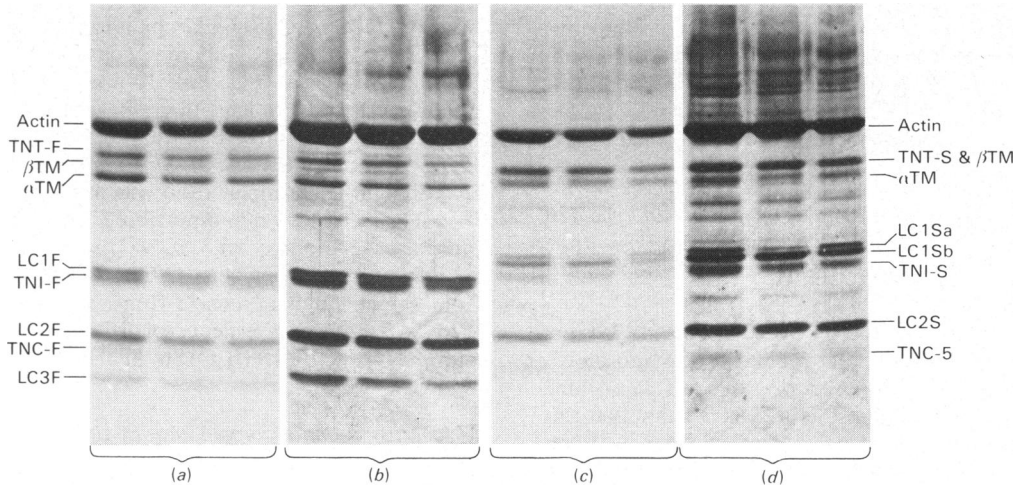

Fig. 2. *Comparison of Coomassie Blue and silver staining methods*

Polyacrylamide-gel electrophoresis of type II (psoas muscle) and type I (soleus muscle) fibres was performed as described in Fig. 1 legend, except that the separating gel was 15% polyacrylamide. After being stained with Coomassie Blue, the gels were photographed, destained and then stained with silver as described in the Materials and methods section. Only the region of low molecular weight is shown. (*a*) Type II fibres stained with Coomassie Blue; (*b*) type II fibres stained with silver; (*c*) type I fibres stained with Coomassie Blue; (*d*) type I fibres stained with silver. Key: as in Fig. 1.

Table 1. *Comparison of the stoicheiometry of myosin light chains of single type II and type I fibres from rabbit skeletal muscles after Coomassie Blue and silver staining*

Polyacrylamide-gel electrophoresis of type II fibres from psoas muscle and of type I fibres from soleus muscle were performed as described in Fig. 2 legend. After electrophoresis, the gel was stained with Coomassie Blue and photographed. The gel was destained with 50% methanol/12% acetic acid solution and then stained with silver as described in the Materials and methods section. Densitometric measurements were made by the method of Volpe *et al.* (1981). Mean values ± S.E.M. for ten fibres of each type are given.

	Portion of individual myosin light chain (%)		
Myosin light chain	Coomassie Blue staining	Silver staining	Silver/Coomassie Blue staining ratio
Type II fibres			
LC1F	35.3 ± 1.6	25.1 ± 1.2	0.7
LC2F	49.1 ± 0.9	54.8 ± 2.3	1.1
LC3F	15.7 ± 1.8	20.2 ± 1.8	1.3
Type I fibres			
LC1Sa	14.4 ± 2.3	18.0 ± 2.2	1.3
LC1Sb	42.1 ± 2.9	38.7 ± 1.8	0.9
LC2S	42.3 ± 2.9	43.3 ± 0.9	1.0

of the respective densitometric areas of myosin light chains with that of tropomyosin. Thus it was found that the myosin light chain/tropomyosin ratio, which was 0.4:1 after staining with Coomassie Blue, increased to values of 1.1–1.3:1 in individual fibres from psoas and soleus muscle. Table 1 shows that the percentage values of fast-twitch-fibre myosin light chains obtained for psoas fibres after staining with Coomassie Blue are in excellent agreement with those reported by Lowey & Risby (1971). On the other hand, a decrease in the percentage values of LC1F light chain and a concomitant increase of LC3F light chain were found after staining with silver. The percentage of the LC1Sa light-chain

component of slow-twitch soleus fibres, on the other hand, increased relatively little, under the same conditions, with silver. Under the same conditions, an increase of the percentage of the LC1Sa light-chain component of slow-twitch soleus fibres was also found.

Stoicheiometry of myosin light chains in type IIB (fast-twitch) and type I (slow-twitch) fibres from predominantly fast-twitch-fibre and predominantly slow-twitch-fibre muscles

It had been reported by Weeds *et al.* (1975) that the central portion of rabbit psoas muscle is homogeneously composed of type IIB fast-twitch fibres. However, the results in Fig. 3 show that this fibre population is widely heterogeneous with respect to relative amounts of myosin LC1F and LC3F light chains. Analysis of the frequency distribution of the LC3F/LC2F light-chain molar ratio among these fibres (Fig. 4*a*) confirms a wide range of variability and no major grouping with respect to this LC3F/LC2F light-chain ratio. As reported previously (Pinter *et al.*, 1981; Julian *et al.*, 1981), a marked degree of variability was also observed in type I slow-twitch fibres from soleus muscle (Fig. 5) with respect to the stoicheiometry of the myosin LC1Sa and LC1Sb light chains, which are regarded to be homologous with the alkali light chains of fast-twitch fibres. Furthermore, we found that the range of variability of myosin LC1Sa/LC1Sb light-chain ratio among type I slow-twitch fibres differed considerably from muscle to muscle (Fig. 4*b*). It was, for instance, much greater for soleus than for crureus muscle fibres, which all showed a marked prevalence of LC1Sb light chain (Figs. 5*i*–5*k*). The mean LC1Sa/LC1Sb light-chain molar ratio was found to be 0.47 ($n = 44$) in slow-twitch-type fibres from the latter muscle as compared with 0.79 ($n = 52$) in corresponding fibres from soleus. Conversely, in fibres from semitendinosus muscle (Figs. 5*g*, 5*h* and 4*b*) there was a clear prevalence of LC1Sa light chain, with a mean LC1Sa/LC1Sb light-chain ratio of 1.14 ($n = 36$).

Myosin light-chain composition of single fibres from mixed muscles

Analysis of myosin light-chain composition in muscle fibres from rabbit mixed skeletal muscles, such as the diaphragm, masseter, vastus lateralis and gastrocnemius, showed three main patterns: (i) fibres with fast-twitch-fibre-type myosin light chains only; (ii) fibres with slow-twitch-fibre-type myosin light chains only; (iii) fibres with a mixed pattern of myosin light chains. The relative proportions of the three main types of fast-twitch, slow-twitch and mixed fibres in the several muscles studied are reported in Table 2. Further heterogeneity within the fast-twitch fibre population relates to the distinction of IIB (fast-twitch white) and IIA (fast-twitch red)

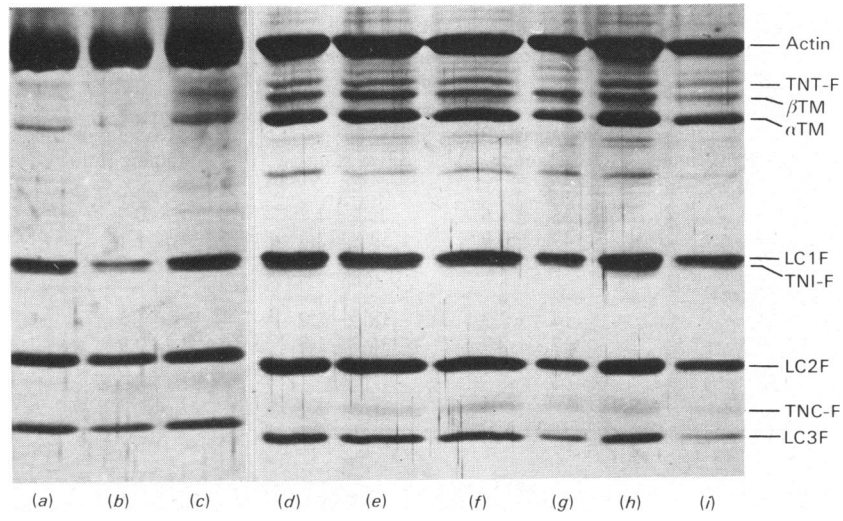

Fig. 3. *Heterogeneity of the stoicheiometry of myosin light chains in type II fibres from predominantly fast-twitch-fibre muscles*

Polyacrylamide-gel electrophoresis of type IIB fibres from gastrocnemius (*a*–*c*) and psoas (*d*–*i*) muscles was performed, in separate experiments, as described in Fig. 2 legend. Only the region of low molecular weight is shown. Key: as in Fig. 1.

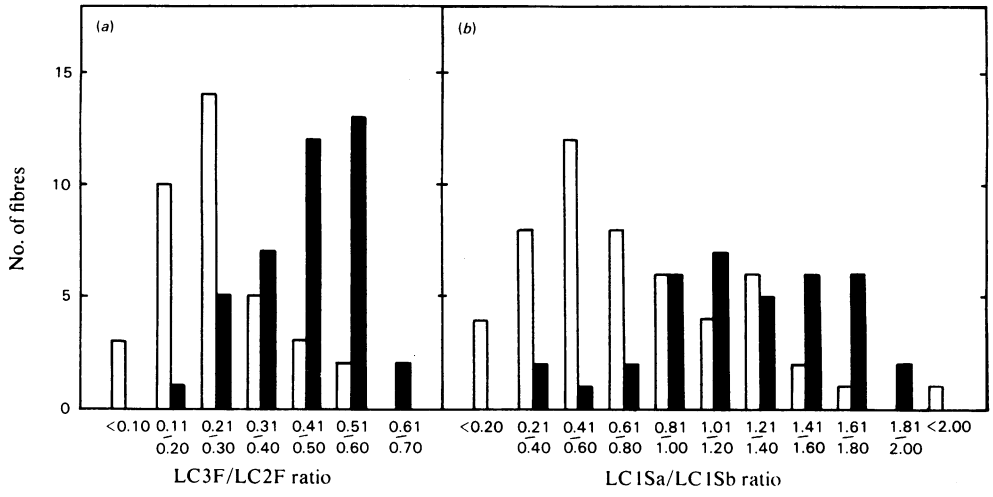

Fig. 4. *Frequency distribution of the myosin LC3F/LC2F light-chain molar ratio in type II fibres (a) and of the LC1Sa/LC1Sb light-chain molar ratio in type I fibres (b)*

Polyacrylamide-gel electrophoresis and silver staining were performed as described in the Materials and methods section. Densitometric measurements were made by the method of Volpe *et al.* (1981). (*a*) Type II fibres from: ■, psoas and gastrocnemius muscle; □, diaphragm, masseter and vastus lateralis muscle. (*b*) Type I fibres from: □, soleus muscle; ■, semitendinosus muscle.

sub-types of these fibres. The latter type of fibres was found to be predominant in muscles such as the diaphragm, vastus lateralis and masseter muscles, whereas fast-twitch fibres from gastrocnemius muscle were virtually all type IIB fibres. As shown in Figs. 6(*c*), 6(*d*), 6(*i*) and 6(*m*), the pattern of myosin light chains of fast-twitch fibres from both the diaphragm, masseter and vastus lateralis muscles was characterized by a smaller proportion of LC3F light chain, as compared with fast-twitch fibres from psoas muscle (cf. Figs. 3*d*–3*i*), in agreement with the immunological results obtained by Gauthier & Lowey (1979). Analysis of the distribution of myosin LC3F/LC2F light-chain molar ratio (Fig. 4*a*) indicates that fast-twitch fibres from mixed muscles are characterized by an average ratio of 0.25 as compared with a value of 0.45 found for psoas as well as gastrocnemius muscles.

With regard to type I slow-twitch fibres, in the fibres from diaphragm, gastrocnemius and vastus lateralis muscles the LC1Sa/LC1Sb light-chain ratio of the constituent myosin was found to vary considerably, similarly to what was observed in predominantly slow-twitch muscles (see above), but with a marked shift towards the lower values. The same type of fibres from the masseter generally lacked the LC1Sa light-chain component, although in the masseter from one rabbit a very small bundle of type I fibres was identified, which contained also this myosin light-chain component. Similarly to hind-limb muscles, the diaphragm and masseter contained a population (5%) of type IIC fibres, with a mixed light-chain pattern, though with characteristically small proportions of both LC3F and LC1Sa light chains (Figs. 6*a*, 6*b*, 6*e* and 6*n*). Furthermore, a distinct population of fibres (about 5% of total fibres) was present in these muscles, as well as in soleus muscles, with a peculiar myosin light-chain pattern. This pattern (Figs. 5*a* and 6*l*) was characterized by the presence of LC1F, LC2F and LC3F light chains in association with LC1Sb light chain, with isoelectrofocusing properties identical with those of the corresponding light chain of type I slow-twitch fibres (not shown). Neither LC1Sa nor LC2S light-chain component was present in the electrophoretograms of these fibres. These fibres were histochemically indistinguishable from type IIA fast-twitch red fibres, with the pure fast-twitch-fibre myosin light-chain pattern. The further evidence in Fig. 7 indicates that, independent of these differences in the myosin light-chain pattern, type IIA fibres can be distinguished from type IIB fibres, as well as from type I fibres, by the peptide composition of the myosin heavy chains, after partial digestion with *Staphylococcus aureus* V_8 proteinase.

Isoforms of regulatory proteins in relation to fibre types

In agreement with the extensive immunological

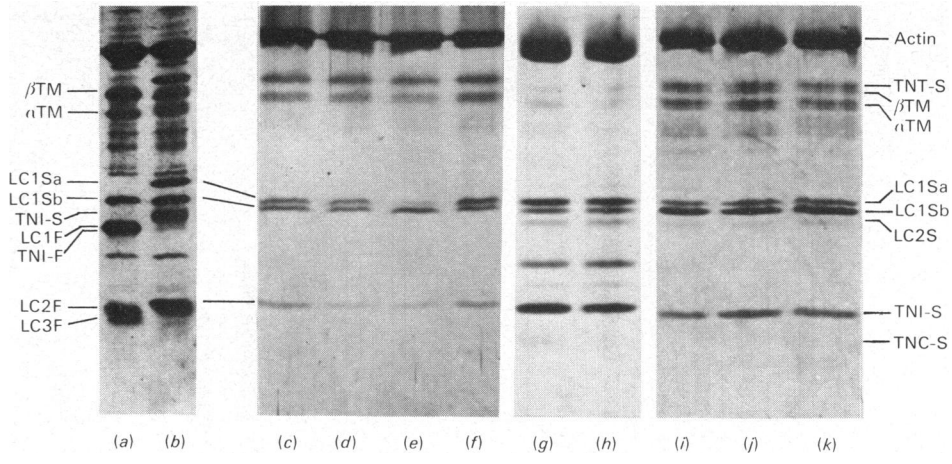

Fig. 5. *Heterogeneity of the stoicheiometry of myosin light chains in type I fibres from predominantly slow-twitch-fibre muscles*

Single muscles fibres were isolated from soleus, semitendinosus and crureus muscles. Polyacrylamide-gel electrophoresis and silver staining were performed as described in the Materials and methods section. The separating gel was 10–20% polyacrylamide linear gradient (tracks *a* and *b*) or 15% polyacrylamide (tracks *c*–*k*). Only the region of low molecular weight is shown. (*a*) Type IIA soleus fibre, showing a hybrid pattern of myosin light chains (LC1F/LC2F/LC3F/LC1Sb); (*b*)–(*f*) type I soleus fibres; (*g*) and (*h*) type I semitendinosus fibres; (*i*) and (*k*) type I crureus fibres. Key: as in Fig. 1.

Table 2. *Distribution of fibre types among rabbit skeletal muscles according to the myosin light-chain pattern*

Fibre typing was performed according to the myosin light-chain pattern after one-dimensional polyacrylamide-gel electrophoresis and silver staining. The slow-twitch-fibre type is the class corresponding to histochemical type I fibres. The fast-twitch-fibre type is the class comprising type IIA (fast-twitch red) and IIB (fast-twitch white) fibres. The intermediate or hybrid type is the class comprising histochemical type IIC (intermediate) fibres and a small percentage of histochemical type IIA fibres (about 5%; see the text).

			Myosin light-chain pattern (%)		
Muscle	No. of fibres	Type of myosin light chains ...	Slow-twitch-fibre type	Fast-twitch-fibre type	Intermediate or hybrid type
Psoas	243		—	100	—
Gastrocnemius	20		10	90	—
Vastus lateralis	29		31	62	7
Diaphragm	243		37	57	6
Masseter	113		55	31	14
Crureus	56		100	—	—
Semitendinosus	47		100	—	—
Soleus	305		94	1	5

studies by Dhoot & Perry (1979), type I fibres and type IIA and IIB fibres, in all muscles investigated, contained either the slow-twitch-fibre or the fast-twitch-fibre isoforms of troponins I, C and T. In type IIC fibres, with the mixed light-chain pattern, however, the coexistence of fast-twitch-fibre and slow-twitch-fibre isoforms of the troponin subunits was occasionally seen (Fig. 6*n*), whereas the other type IIC fibres showed the presence of the fast-twitch-fibre troponin isoforms only. That was also the case of the subtype of type IIA fibres having a myosin LC1F/LC2F/LC3F/LC1Sb light-chain pattern (Fig. 6*l*).

With regard to the distribution of the α- and β-tropomyosin subunits among the different types of fibres, that observed in type IIB fibres was identical with that reported for the isolated tropomyosin from rabbit fast-skeletal-twitch skeletal muscles, i.e. with

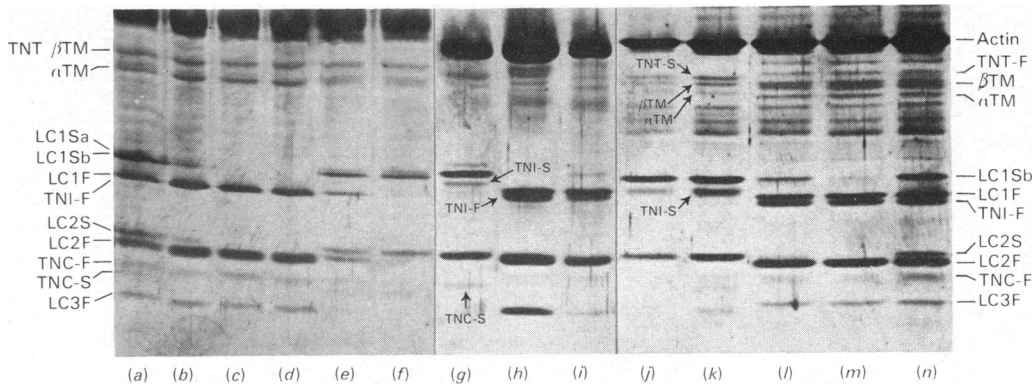

Fig. 6. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoretic pattern of myosin light chains and of regulatory proteins of fibres from mixed muscles*

Polyacrylamide-gel electrophoresis and silver staining were performed as described in Fig. 2 legend. Only the region of low molecular weight is shown. Representative fibres from each muscle were from separate experiments. (*a*)–(*f*) Diaphragm muscle; (*g*)–(*i*) vastus lateralis muscle; (*j*)–(*n*) masseter muscle. Key; as in Fig. 1.

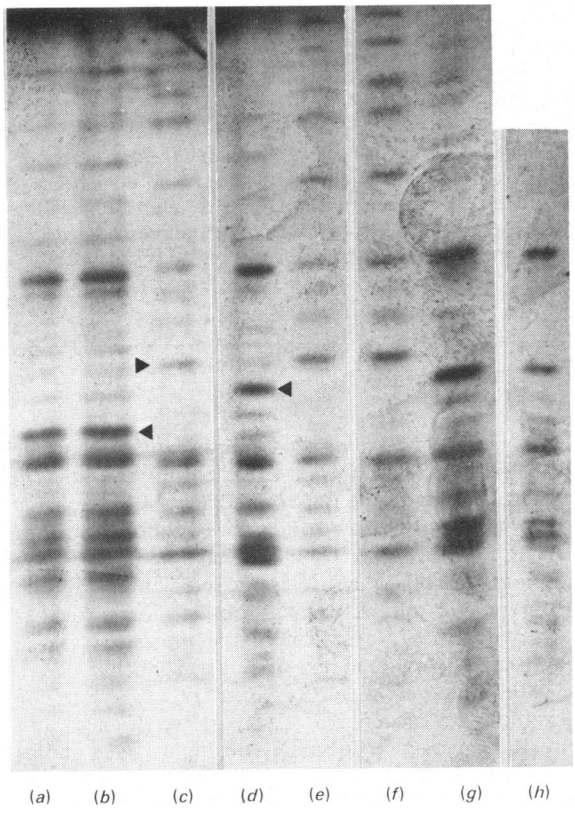

Fig. 7. *Peptide 'mapping', after digestion with Staphylococcus aureus V_8 proteinase, of myosin heavy chains of single type IIB, type IIA and type I fibres from rabbit skeletal muscles*

Purification of myosin heavy chains of single muscle fibres, as well as the digestion with *Staphylococcus aureus* V_8 proteinase, were performed as described in the Materials and methods section. Tracks (*a*)–(*e*) were from the same gel, and tracks (*f*) and (*g*) and track (*h*) from separate experiments. (*a*) and (*b*) Type IIB fibres from psoas muscle; (*c*) and (*e*) type I fibres from soleus muscle; (*d*) type IIA fibre from soleus muscle; (*f*) type I fibre from diaphragm muscle; (*g*) type IIA fibre from diaphragm muscle; (*h*) type IIA fibre from masseter muscle. Arrows indicate peptides characteristic of each type of myosin heavy chains.

predominance of the α-subunit. As determined by densitometric measurements the α-/β-tropomyosin ratio was 4:1 in these fibres. In type I fibres the α-/β-tropomyosin ratio was 9:11. These results are in agreement with previous results obtained by Roy *et al.* (1979). However, in the electrophoretograms of type I fibres α-tropomyosin appeared as a doublet instead of a single component (Figs. 1 and 8). This appearance could not be explained by the occurrence of some modification of the tropomyosin subunit during chemical skinning or storage. Furthermore, formation of a doublet of the α-tropomyosin subunit was observed also with tropomyosin purified from rabbit slow-twitch muscles (Fig. 8).

The results of electrophoresis of single type I fibres indicate a considerable variability in the relative amounts of the two components of the α-tropomyosin subunit (Fig. 8). A similar splitting of the α-subunit was observed in some of the type IIA fibres of masseter (Figs. 6*l* and 6*m*) and psoas muscles. Circumstantial evidence indicates, however, that the subunit components of lower mobility in the latter type of fibres are not identical with its type I slow-twitch-fibre counterpart.

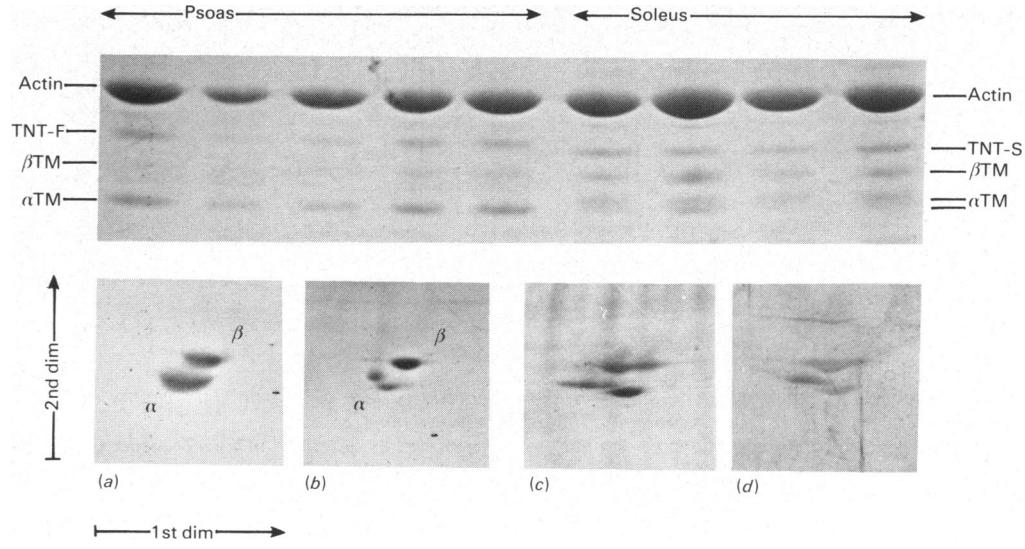

Fig. 8. *Tropomyosin subunit composition of type II and type I muscle fibres and of purified tropomyosin from fast-twitch and slow-twitch rabbit muscles*

Tropomyosin was purified from rabbit fast-twitch (back and leg muscle) and slow-twitch (soleus, semitendinosus and crureus) muscles by the method of Roy *et al.* (1979). Upper panel: one-dimensional polyacrylamide-gel electrophoresis of type IIB fibres (psoas muscle) and type I fibres (soleus muscle) was performed as described in Fig. 1 legend. After electrophoresis, the gel was stained with Coomassie Blue. Only the actin–tropomyosin region is shown. Lower panel: two-dimensional electrophoresis of purified tropomyosin (2 μg of protein) from rabbit fast-twitch (*a*) and slow-twitch (*b*) muscles and of type I fibres from soleus (*c*) and type I fibres from diaphragm (*d*) muscles. Isoelectrofocusing (first dimension) and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (second dimension) were performed as described in the Materials and methods section. After electrophoresis the gels were stained either with Coomassie Blue (*a* and *b*) or with silver (*c* and *d*). Only the tropomyosin region is shown.

Discussion

The present study shows that high-resolution one-dimensional gel electrophoresis, combined with a highly sensitive silver staining method, is a powerful tool for studying the distribution of the several isoforms of myofibrillar proteins among single fibres. The method of isolation of muscle fibres used in the present work, involving chemical skinning of the fibres, in itself appears to be superior to other methods (see, e.g., Young & Davey, 1981), in that it accomplishes an effective removal of soluble sarcoplasmic proteins.

Our results show that rabbit skeletal-muscle fibres can be classified, according to the myosin light-chain pattern, into fibres with (i) an 'exclusively' slow-twitch-fibre (LC1Sa/LC1Sb/LC2S) light-chain pattern or (ii) a fast-twitch-fibre (LC1F/LC2F/LC3F) light-chain pattern or (iii) a mixed light-chain pattern.

Fibres belonging to category (i), and that are all histochemically classified as type I fibres on the basis of staining for myosin ATPase activity, can be further subdivided into two main subtypes, i.e. (*a*) fibres containing the full complement of light chains characteristic of slow-twitch-fibre myosin (LC1Sa/LC1Sb/LC2S) and (*b*) fibres lacking the LC1Sa light-chain component (e.g. masseter fibres). Within subtype (*a*) of slow-twitch-muscle fibres, further heterogeneities are shown in the present work, in agreement with previous findings (Schachat *et al.*, 1980; Pinter *et al.*, 1981; Biral *et al.*, 1982), for example the existence of a wide range of variability in the myosin LC1Sa/LC1Sb light-chain molar ratio. Among these fibres, at the same time, our results demonstrate that, in spite of these differences in stoicheiometry of the light chains, the type of heavy chains associated with slow-twitch-fibre myosin is invariably the same, on the basis of the peptide 'map' obtained after digestion of the heavy chains with *Staphylococcus aureus* V_8 proteinase. Further, in agreement with the immunological data of Dhoot & Perry (1979), our electrophoretic data show that

slow-twitch fibres are characterized by a unique set of isoforms of the three troponin subunits. The present results, however, indicate that slow-twitch fibres do not contain exclusively the β-tropomyosin subunit, but also a tropomyosin subunit with electrophoretic mobility similar to, but not identical with, that of the α-subunit of tropomyosin, which according to Dhoot & Perry (1979) is specific to fast-twitch fibres. Because of the apparent discrepancy between our electrophoretic results and the immunological findings reported by Dhoot & Perry (1979), it would be tempting to assume that the 'α-like' subunit component that we found to be present in rabbit slow-twitch fibres is antigenically different from the 'true' α-subunit of tropomyosin. That would suggest a greater polymorphism of rabbit muscle tropomyosin than had been previously thought, i.e. two (fast-twitch-fibre and slow-twitch-fibre) isoforms of α-tropomyosin besides β-tropomyosin. On the other hand, heterogeneity of the α-tropomyosin subunit of fast-twitch and slow-twitch muscles has been reported for cat (Steinbach *et al.*, 1980) and chicken (Montarras *et al.*, 1981) muscles.

As far as fast-twitch fibres are concerned, a wide range of values in the myosin LC3F/LC2F light-chain ratio, analogous to that described for the myosin LC1Sa/LC1Sb light-chain ratio, is observed within this population of fibres of the same muscle, as well as in comparisons of different muscles. This result is in agreement with comparative data on the light-chain composition of the isolated myosin from whole muscle (Biral *et al.*, 1982). Our present data further demonstrate that this heterogeneity is basically linked to the existence of two sub-types of fast-twitch fibres, corresponding to subtypes IIA and IIB respectively. Thus type IIA fast-twitch red fibres, which are much more numerous in muscles such as the masseter than in predominantly fast-twitch-fibre psoas muscle, are characterized by a myosin composition with a very low proportion of the LC3F light chain. A further distinguishing feature of fast-twitch red fibres is that a unique type of heavy chains, i.e. different from the types found in fast-twitch white and the slow-twitch fibres, appears to be associated with the myosin of these fibres. This finding therefore implements earlier suggestions (Dalla Libera *et al.*, 1980). If the type of myosin heavy chains can be used as a unifying criteria for classifying fast-twitch red fibres, it would, however, follow from the additional electrophoretic data reported here that the light-chain pattern of the constituent myosin can be either a pure fast-twitch-fibre light-chain pattern, as above described, or a hybrid LC1F/LC2F/LC3F/LC1Sb light-chain pattern. Notably, the value found for the (LC1Sb + LC3F)/LC2F light-chain ratio in these fibres appears to be similar to the LC3F/LC2F light-chain ratio in the predominant type of fast-twitch red fibres. That may suggest that these hybrid forms of myosin relate to the existence of LC1F–LC1Sb myosin heterodimers. Fast-twitch red fibres with the hybrid form of myosin with respect to the light chains account for about 10% of total fast-twitch red fibres in several muscles examined. The LC1Sb light chain associated with the myosin of these fibres is electrophoretically indistinguishable from the LC1Sb light-chain component of myosin of type I slow-twitch fibres. However, recent results have shown that the isolated LC1Sb light chain from the myosin from rabbit masseter, where fast-twitch red fibres predominate, is antigenically different from its slow-twitch-fibre counterpart (Biral *et al.*, 1982). The suggestion that a unique type of the LC1Sb light chain may be associated with myosin of fast-twitch red fibres (Biral *et al.*, 1982) fits with the observation that myosin (LC1F/LC2F/LC1Sb) hybrids appear to be specific to these fibres, as shown in the present work. On the other hand, truly intermediate fibres, i.e. fibres that are histochemically classified as type IIC fibres, have a truly mixed myosin light-chain pattern, i.e. with coexistence of all the light chains characteristic of type I and type II fibres. Furthermore, type IIC fibres are similarly promiscuous with respect to the isoforms of troponin, whereas type IIA fast-twitch red fibres have a subset of troponin subunits indistinguishable from that of type IIB fast-twitch white fibres.

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