Further studies on single fibres of bovine muscles

Owen A. YOUNG

Meat Industry Research Institute of New Zealand (Inc.), P.O. Box 617, Hamilton, New Zealand

(Received 19 October 1981/Accepted 15 December 1981)

Young & Davey (1981) (*Biochem. J.* **195**, 317–327) identified numbers of polymorphs of myofibrillar proteins by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of single muscle fibres isolated from three bovine muscles. Fibres were classed according to the distribution of polymorphs. The study has now been extended to eight diverse bovine muscles. The previous distinction made between fast and slow fibres is valid without exception in the extended study. Within these classes, variations in myofibrillar expression are examined within and between fibres, muscles and animals. Two slow muscles are contrasted; masseter is homogeneous in fibre type, whereas diaphragm is subtly heterogeneous, possibly arising from greater physiological demands. Of the myofibrillar polymorphs, attention is concentrated on two variants of fast-muscle myosin heavy chain. Both are present in all fast and mixed muscles examined, except sternomandibularis, and each is respectively associated with certain unidentified proteins. Within a muscle the fast-muscle myosin light-chain expression is the same irrespective of the heavy-chain variant. Histochemical techniques demonstrated that the variants are respectively associated with types IIA and IIB as defined by other investigators.

In a recent study, individual muscle fibres from three bovine muscles were analysed by SDS/polyacrylamide-gel electrophoresis (Young & Davey, 1981). When stained with Coomassie Blue the resulting electrophoretic patterns were of sufficient density and resolution to identify several polymorphs of myofibrillar proteins. Inspection of these patterns, coupled with peptide mapping (Cleveland *et al.*, 1977) of gel zones containing myosin heavy chain, led to a classification scheme which differentiated slow and fast fibres, often called types I and II respectively (Peter *et al.*, 1972). All fibres examined contained an α - and a β -tropomyosin (Cummins & Perry, 1973), but two forms of α -tropomyosin were distinguished, one associated with fast fibres and one with slow. Fast and slow fibres were further classed according to the distribution of their myosin light chains and troponins.

The single-fibre analysis technique has now been extended to five other bovine muscles representing a wide range of muscle functions. In the present paper the fibre characteristics from these five muscles are examined in relation to those from the original three muscles, and the peculiarities of certain muscles are discussed. Further, the relationships of

two fast myosin heavy-chain variants to fibre types classically identified by histochemistry are examined.

Experimental

Dissection of muscles

The muscles examined are listed in Table 1. Longissimus dorsi (ld), which runs dorsally the

Table 1. Sources of bovine muscle fibres

The fibres were isolated as described in the text. Of the grand total of 665 fibres, 167 from sternomandibularis, masseter and rectus abdominis were examined in a previous study (Young & Davey, 1981).

Muscle	No. of animals sampled		Total no. of fibres
	Bull	Ox*	
Sternomandibularis	10	0	147
Masseter	5	1	43
Rectus abdominis	0	6	63
Longissimus dorsi	3	8	189
Psoas major	0	3	55
Cutaneus trunci	0	2	33
Diaphragm	1	5	87
Semimembranosus	0	2	48

* Here and elsewhere this means a castrated bull.

Abbreviations used: SDS, sodium dodecyl sulphate; ld, longissimus dorsi.

length of the backbone, was sampled from the core of the muscle in the region of the 13th (last) rib. Psoas major, which lies ventral to the vertebrae in the lower back, was core-sampled in the middle of the lumbar region. Cutaneus trunci, the lateral superficial muscle sheet that twitches the skin, was sampled in the lower flank. The semimembranosus, a large muscle on the inside back leg, was sampled near the mid-point of the shaft of the femur. Diaphragm, the principal muscle of breathing, was sampled in the costal region. Also, additional examples of the three muscles studied by Young & Davey (1981) were sampled.

Fibre bundles from fresh bovine muscles were teased apart in phosphate-buffered saline (137 mM-NaCl/3 mM-KCl/2 mM-KH₂PO₄/8 mM-Na₂HPO₄, pH 7.3) containing 5 mM-EGTA, using a stereomicroscope, as described previously (Young & Davey, 1981). The fibres were transferred to miniature test tubes and kept frozen until needed for analysis.

SDS/polyacrylamide-gel electrophoresis and peptide mapping

Gel analyses were performed as previously described (Young & Davey, 1981). Unless otherwise stated the resolution gels contained 12.5% (w/v) acrylamide, 1.2% cross-linked. Gels were stained either conventionally with Coomassie Blue, or with silver stain (Oakley *et al.*, 1980) after peptide mapping. Myofibrillar proteins of single fibres were identified on gels by comparison with authentic standards purified from the same muscle tissue (Young & Davey, 1981).

Peptide mapping of heavy-chain myosin purified on gels was performed as described by Cleveland *et al.* (1977) with papain type IV and chymotrypsin (type II) from Sigma Chemical Co., and *Staphylococcus aureus* (V8) proteinase from Miles Laboratories.

Histochemistry

Muscle blocks (0.5 cm sides) were frozen in isopentane cooled to freezing in liquid N₂. Cross-sections (10 µm thick) were cut on an Ames cryostat at -18°C, and myofibrillar ATPase activity in these cross-sections was examined. Activity was measured at pH 9.4, after preincubation in formaldehyde fixative, as described by Guth & Samaha (1969). Under these conditions types IIB (glycolytic), IIA (oxidative-glycolytic) and I stain dark, intermediate and light respectively (Peter *et al.*, 1972; Hunt & Hedrick, 1977).

Measurements of the number of a particular fibre type were made by a transect technique on optical micrographs. The areas of fibres struck by the transect line were measured by a cut-and-weigh technique.

Results and discussion

A reassessment of muscle fibre types

Young & Davey (1981) classified each of 167 fibres from sternomandibularis, masseter and rectus abdominis muscles into one of two broad groups, fast and slow, on the basis of the mobility of their proteins on 12.5%-acrylamide gels. In the present analysis of 498 more fibres, this classification continued to be valid without exception. Thus the classical distinction between type I and type II fibres is convincingly corroborated by electrophoretic analysis.

Three broad categories of fast fibres were described by Young & Davey (1981) (Scheme 1). Minor variations to these three categories were observed in the present study. In some muscles, troponin I fast was present as a doublet in fibres which would otherwise be classed as fast₁ based on myosin light-chain expression (a definitive feature to the eye on these gels). Conversely, troponin I fast was sometimes a singlet in fibres which would otherwise be fast₂ fibres. If myosin light chains are considered the most important feature in classification, then fast₁ was the most common fast fibre type, all muscles considered (Table 2), and this is the well-known arrangement first described by Lowey & Risby (1971) (Fig. 1a). Fast₃ was demonstrated in fibres from a single muscle (Young & Davey, 1981) and presumably represents a rare or transient physiological state. All other fast fibres were fast₂. Pette *et al.* (1979), who studied human muscle, observed two myofibrillar proteins in the

Scheme 1. *An outline of fast fibre classification*
The key characteristics of three fast fibre types, as seen on 12.5%-polyacrylamide gels, are summarized. The bars (————) approximately locate the relative migrations of the three myosin light chains (LC1_f, etc.) and the fast troponin components I and C (TI_f, TC_f) associated with each type. In fast₂ and fast₃ the migration of one of the light chains was more typical of myosin light chain 1 of slow fibres, and so is labelled 'LC1_s?'. A broken bar (-----) indicates variable expression of a protein, and (2) means a protein is bimorphic.

Table 2. *Distribution of fast fibre types among the muscles*

In this analysis the myosin light-chain patterns, outlined in Scheme 1, have been considered the most important feature in fast-fibre classification. The 'unknown' category comprises muscles where the fibres were analysed on a gel that did not display the light chains, and muscles where no fast fibres were encountered but were very likely present. Masseter has not been included because it is homogeneously slow (Young & Davey, 1981). Fast₁ was the most common fast fibre type.

Muscle	Numbers of examples of:			
	Fast ₁	Fast ₂	Fast ₃	Unknown
Sternomandibularis	6	3	1	0
Rectus abdominis	5	0	0	1
Longissimus dorsi	7	1	0	3
Psoas major	2	1	0	0
Cutaneus trunci	1	1	0	0
Diaphragm	2	0	0	4
Semimembranosus	2	0	0	0
Totals	25	6	1	8

vicinity of myosin light chain 1 fast on SDS/polyacrylamide-gel electrophoresis of single fast fibres. Fast₂ probably represents the bovine equivalent of this configuration.

The staining patterns of slow fibres were less variable than those of fast fibres, but three types were distinguished by Young & Davey (1981). In the present study a continuum of subtle variation in slow-fibre patterns was encountered, which defied simple classification.

Staining-pattern variations within and between fibres, muscles and animals

At any one sampling point in any one muscle a small number of distinct staining patterns could be recognized. Fibres from the same muscle, but from another animal, often showed the same number of distinct patterns, each of which had an obvious equivalent in the first muscle, but the equivalents were seldom identical. Equivalent fibres could often be recognized between muscles within one animal, but again were seldom identical.

Fig. 1. *Some features of bovine fast fibres*

(a) The two fast myosin heavy-chain variants, A and B, were present in all the fast and mixed muscles examined, except in sternomandibularis, where only variant A was found. On 12.5%-polyacrylamide gels, certain protein bands and clusters of bands were always associated with the respective variants, and the more obvious of these are marked with arrows. Myosin heavy-chain (MHC) zones have been cut out for peptide mapping. Identities of proteins were assigned as described in Young & Davey (1981). Relevant abbreviations are defined in Scheme 1 and as follows: cu, cutaneous; sm, sternomandibularis; ld, longissimus dorsi. Within each of most of the fibres isolated, α - and β -tropomyosin (α -TM, β -TM) were present in equal amounts. However, in one ld muscle the amount of β -tropomyosin was considerably decreased (fibres 8 and 9). By reference to Scheme 1 and the text, each of the nine fibres may be classed as fast₁ or fast₂. (b) The two fast myosin heavy-chain variants could be identified by peptide mapping with any of three proteinases. The four peptide maps shown here are of myosin heavy-chain zones from fibres 1 and 2 (variant B) and 3 and 4 (variant A). The fifth track is a no-substrate control. Each track on this 15%-acrylamide gel (3% cross-linked) contained 0.4 μ g of chymotrypsin and 0.2 μ g of egg-white trypsin inhibitor.

At different sampling points within a muscle of one animal, identical staining patterns were often obtained. For example, in semimembranosus, the fast fibres isolated from within one muscle had an identical staining pattern, whether sampled on the inner side or the outer side, some 10 cm apart. (But there were subtle variations between semimembranosus fibres from different animals.) In sternomandibularis a similar result was obtained when fibres were sampled from the core and the periphery (Young & Davey, 1981).

The fact that many fibres with identical staining patterns can be readily isolated from a single muscle has an interesting consequence; protein expression is unchanging throughout the fibre length, since in sampling one usually has little control over which part of a fibre is selected. This constancy within a fibre was formally verified in psoas and ld fibres by analysing different fragments of single fibres in adjacent gel tracks; the pattern within each pair of tracks was identical.

Fibre variation in diaphragm and masseter

Of 87 fibres from six diaphragm muscles, only four were fast. All others were one of two variants of slow fibre. These variants were isolated in approximately equal numbers, and are labelled X and Y (Fig. 2). Monin (1980) reported that, after masseter, diaphragm was the slowest of 18 bovine muscles as measured by myofibrillar ATPase activity. Variants X and Y presumably dominate diaphragm muscle. Two slow muscles can now be contrasted: masseter, the cud-chewing muscle, is slow and homogeneous (Young & Davey, 1981), whereas diaphragm, a breathing muscle, is slow but subtly heterogeneous. The physiological reason for this contrast may relate to a wide range of demands made on the diaphragm during physical activity. In a microscopic study of bovine diaphragm, Gauthier & Padykula (1966) found it to be composed of large white fibres (low in mitochondrial content), but no heterogeneity was reported. Recently, two types of slow fibres were observed in rabbit skeletal muscle (Schachat *et al.*, 1980), so it is likely that more examples of slow-fibre variability will be encountered in other animals.

Expression of some myofibrillar polymorphs

No new polymorphs of the myosin light chains were encountered beyond those described in Young & Davey (1981). The literature contains numerous references to myosin light-chain polymorphs and their combinations, but with the exception of the findings of Pette *et al.* (1979), discussed above, it is unclear how they relate to the present single-fibre studies. No additional polymorphs of fast and slow troponins T, I and C were found. Numerous instances of bimorphic troponin T fast (Young & Davey, 1981) were encountered.

Fig. 2. Slow fibres from the slow muscles, diaphragm and masseter

Slow fibres were isolated from the diaphragm (dp) and the masseter (ma) of a bull. Masseter fibres were all identical, but two variants of slow fibre, X and Y, were isolated from diaphragm. Closely similar fibre types were obtained from the same muscles of oxen (castrated bulls). The homogeneity of masseter and the heterogeneity of diaphragm may relate to a wider range of physiological demands made on diaphragm. At molecular weights above that of β -tropomyosin (β -TM), X and Y are closely similar, but there is variation at lower molecular weights. The identities of slow myosin light chains 1 and 2 ($LC1_s$, $LC2_s$) and the slow troponin components T, I and C (TT_s , etc.) have been assigned for masseter, as detailed in Young & Davey (1981). The identities of these proteins in diaphragm have not been investigated, but variant X and masseter fibres appear qualitatively similar.

All fibres contained an α - and a β -tropomyosin. In each of the majority of fibres isolated, the staining intensities of α - and β -tropomyosins were approximately equal. In view of the close similarity in secondary structure of tropomyosins (Mak *et al.*, 1980), this equal staining intensity indicates they are normally present in equimolar amounts in adult bovine muscle fibres. However, the concentration of β -tropomyosin was much decreased in fast fibres from one ld muscle (Fig. 1a) and was slightly decreased in fast fibres from the two semi-membranosus muscles. Such between-animal and between-muscle variability of α - and β -tropomyosin distribution may be at the root of the confusion that surrounds their expression; from immunological studies on human semispinalis capitis muscle, Dhoot & Perry (1979) proposed that α - and β -tropomyosin were restricted to fast and slow fibres respectively, whereas the single-fibre approach of Bronson & Schachat (1980) with rabbit muscle and the present bovine study indicate there is no such absolute restriction. On the other hand, differences in tropomyosin distribution may be species-related.

The present study focused attention on the two fast myosin heavy-chain polymorphs described by Young & Davey (1981). The two variants, called A and B for the purpose of discussion, occur throughout the fast and mixed muscles examined, except for sternomandibularis, where only variant A was observed in a total of 117 fast fibres from ten animals. The banding patterns respectively associated with the two variants were subtly distinct and differed little from muscle to muscle (Fig. 1a); the component proteins of the bands have not been identified. The two variants were originally identified by peptide mapping with *S. aureus* proteinase. The variants could also be identified by using chymotrypsin (Fig. 1b) or papain, which also

demonstrated only subtle differences between the maps of A and B.

Young & Davey (1981) noted that the variants did not dictate the expression of myosin light chains; in the present study this relationship still holds true. As a corollary, within one muscle from a given animal, the fast-light-chain expression was substantially the same irrespective of the heavy-chain variant.

There is some evidence that the expression of variants A and B in ld muscle is linked to differences arising from castration. From the ld muscles of eight oxen (castrated bulls), 70% of 132 fast fibres contained variant B. By contrast, of 32 fast fibres from three bulls, only two (6%) contained variant B.

Relationship of fast myosin heavy-chain variants to histochemically defined fibre types

Whenever a fibre was described as 'wide' during dissection of ox ld muscle, it was subsequently found to contain variant B of fast myosin heavy chain. Variant B also occurred in some fibres described as 'undistinguished' but never in fibres described as 'narrow'. Type I fibres and fast fibres containing variant A were either narrow or undistinguished. Moreover, fibres most commonly isolated from ox ld muscle contained variant B.

In a histochemical study, Hunt & Hedrick (1977) examined the profile of fibre types in the ld muscle of five oxen, sampled at the same point as in the present study. The fibres with the greatest average cross-sectional area, and thus the widest, were type IIB, fast-twitch glycolytic fibres (Peter *et al.*, 1972). They were also the most common. These findings suggested that, in the present study, fibres containing fast myosin heavy-chain variant B are equivalent to type IIB and, by difference, that fibres containing variant A are equivalent to type IIA, fast-twitch oxidative-glycolytic fibres.

To test this hypothesis within one muscle, 40 fibres were dissected from an ox ld muscle, taking particular care to obtain a spectrum of diameters. Fibres were classed as 'wide', 'undistinguished' or 'narrow', and an approximate measure of diameter at the widest point was obtained with a graticule eyepiece. After SDS/polyacrylamide-gel electrophoresis, fibres were classed into three types on the basis of myosin heavy-chain composition (Table 3). Variant B was clearly associated with wide fibres.

At the same time, blocks of ld muscle sampled from areas adjacent to the single-fibre sampling point were frozen, and cross-sections were stained for myofibrillar ATPase. From a population of 344 fibres the percentage occurrence and average cross-sectional area of each fibre type were determined (Table 4); type IIB fibres were generally wider (greater area) than the others and were more

Table 3. *Size distribution of fibres isolated from an ox ld muscle*

Fibres, 40 in all and isolated from an ox ld muscle, were classed into size categories. Fibres classed as 'wide', 'undistinguished' and 'narrow' were measured at the widest point and had average diameters ($\pm$ s.d.) of 105 ± 13.4 , 82 ± 5.8 and $71 \pm 10.9 \mu\text{m}$ respectively. After analysis on SDS/polyacrylamide gels the three fibre types, classified by myosin heavy-chain composition, were found to be unevenly distributed among the size classes. Variant B was clearly associated with wide fibres.

Types defined by myosin heavy-chain composition	No. of fibres occurring in the three size classes		
	Wide	Undistinguished	Narrow
Type I	0	0	6
Type II			
Variant A	0	3	2
Variant B	15	13	1

Table 4. *Myofibrillar ATPase of ox ld muscle*

Cross-sections of ox ld muscle were cut and stained for myofibrillar ATPase. Despite the high s.d. values, the average area of type IIA fibres was significantly different from that of type IIB, at $P = 0.01$. Type IIB was the most numerous of the 344 fibres measured.

Fibre type (Peter <i>et al.</i> , 1972)	Myofibrillar ATPase stain intensity at pH 9.4	Percentage of total	Average cross-sectional area $\pm$ s.d. (μm^2)
Type I	Light	26.2	2528 $\pm$ 853
Type IIA	Intermediate	25.3	2440 $\pm$ 882
Type IIB	Dark	48.5	3234 $\pm$ 1073

numerous. Comparison of Tables 3 and 4 suggests that variants A and B occur in fibre types IIA and IIB respectively.

Additional evidence was found in sternomandibularis muscle, where none of the 117 fast fibres isolated contained fast myosin heavy-chain variant B. One of these muscles was sectioned in the same way as the ld muscle above and stained for myofibrillar ATPase. There was far less variability in staining intensity within the population of fast fibres than in ld muscle, which contains both variants. Moreover, the staining intensity of the fast population appeared 'intermediate', as expected for variant A (Table 4).

Gauthier & Lowey (1979) found that fast-twitch red and fast-twitch white fibres (apparently IIA and IIB respectively) of rat reacted unequally with antibodies to myosin fragments isolated from type IIB fibres of chicken pectoralis muscle. Dalla Libera *et al.* (1980) have identified two variants of fast myosin heavy chain in guinea-pig muscles; the histochemical profiles of the muscles and immunohistochemical evidence supported this concept of unique heavy-chain determinants in fibre types IIA and IIB. Very recently, Billeter *et al.* (1981) have identified two fast myosin heavy chains, each associated with either fibre type IIA or IIB of human muscle. The present study appears to extend to bovine muscle the association of fast myosin heavy-chain variants with defined histochemical types.

In bovine muscle, fibre types IIA and IIB are defined by differences in myofibrillar ATPase activity at pH 9.4 and NADH:tetrazolium reductase activity (Hunt & Hedrick, 1977). If, as appears very likely, the myosin heavy-chain variants A and B are strictly linked to types IIA and IIB, then the difference in ATPase activity probably arises from a structural difference on the myosin heavy chain, since the light-chain composition of all fast fibres is substantially the same within one muscle.

In fibres where myosin light-chain 3 fast was strongly expressed, glycolytic enzymes were frequently co-expressed (Young & Davey, 1981). This observation led to the apparently erroneous proposal that such glycolytically disposed fibres were type IIB, and, by difference, other fast fibres were type IIA. Rather, in this expanded study the co-expression has been observed to occur freely with myosin

variants A and B. Apparently this variability is a further example of the wide range of metabolic activities associated with fast fibres (Spamer & Pette, 1977; Lowry *et al.*, 1978).

I thank Mrs. Linda Catchside for her technical assistance with part of this work.

References

- Billeter, R., Heizmann, C. W., Howald, H. & Jenny, E. (1981) *Eur. J. Biochem.* **116**, 389–395
- Bronson, D. D. & Schachar, F. H. (1980) *J. Cell Biol.* **87**, 263a
- Cleveland, D. W., Fischer, S. G., Kirschner, N. W. & Laemmli, U. K. (1977) *J. Biol. Chem.* **252**, 1102–1106
- Cummins, P. & Perry, S. V. (1973) *Biochem. J.* **133**, 765–777
- Dalla Libera, L., Satoro, S., Pierobon-Bormioli, S. & Schiaffino, S. (1980) *Biochem. Biophys. Res. Commun.* **96**, 1662–1670
- Dhoot, G. K. & Perry, S. V. (1979) *Nature (London)* **278**, 714–718
- Gauthier, G. F. & Lowey, S. (1979) *J. Cell Biol.* **81**, 10–25
- Gauthier, G. F. & Padykula, H. A. (1966) *J. Cell Biol.* **28**, 333–354
- Guth, L. & Samaha, F. J. (1969) *Exp. Neurol.* **25**, 138–152
- Hunt, M. C. & Hedrick, H. B. (1977) *J. Food Sci.* **42**, 513–517
- Lowey, S. & Risby, D. (1971) *Nature (London)* **234**, 81–85
- Lowry, C. V., Kimmey, J. S., Felder, S., Chi, M. M.-Y., Kaiser, K. K., Passonneau, P. N., Kirk, K. A. & Lowry, O. H. (1978) *J. Biol. Chem.* **253**, 8269–8277
- Mak, A. S., Smillie, L. B. & Stewart, G. R. (1980) *J. Biol. Chem.* **255**, 3647–3655
- Monin, G. (1980) in *The Problem of Dark-Cutting in Beef (European Economic Community Seminar, Brussels, 7–8 October 1980)*, in the press
- Oakley, B. R., Kirsch, D. R. & Morris, N. R. (1980) *Anal. Biochem.* **105**, 361–363
- Peter, J. B., Barnard, R. J., Edgerton, V. R., Gillespie, C. A. & Stempel, K. E. (1972) *Biochemistry* **11**, 2627–2633
- Pette, D., Henriksson, J. & Emmerich, M. (1979) *FEBS Lett.* **103**, 152–155
- Schachar, F. H., Bronson, D. D. & McDonald, O. B. (1980) *FEBS Lett.* **122**, 80–82
- Spamer, C. & Pette, D. (1977) *Histochemistry* **52**, 201–216
- Young, O. A. & Davey, C. L. (1981) *Biochem. J.* **195**, 317–327