Histochemical and Structure-Biological Quantification of Muscle Physiological Properties

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Quantitative Immunohistochemical Method for Assessment of Physiological Properties of Skeletal Muscles

Conventional histochemical classification of muscle fiber types

Histochemical methods for classification of myofiber type have been used to research the physiological properties of skeletal muscles of wild vertebrates (amphibians^{60,61}, reptiles⁶², birds⁶³, mammals^{59,118,126,127,129,131}, etc.) and experimental animals^{1-3,103}. The histochemical myofiber type composition of each skeletal muscle reflects its function in locomotion and energy metabolism^{10,11,13,55,99,100,120}. Many techniques have been developed for evaluation of meat quality in carcasses of farm animals, e.g. ultrasonography, fiber-optic spectrophotometry, etc.³⁶⁻⁴³. Histochemical myofiber types have been investigated in domestic fowls (chickens^{44,46-51,53,92-96,119}, Guinea fowls⁶⁹, etc.) and animals (pigs^{8,34,45,97}, cattle^{9,24-26,32,52,64,67,68,98,112-116}, sheep^{109-111,121,128,130}, goats^{33,80,81,122-125,132,133}, horses^{124,125}, etc.), and myofiber classification is considered to be es-

sential for precise evaluation of meat quality based on its physiological properties^{14,21,54,85,115}. For instance, lipids are stored mainly in type I fibers (slow-twitch oxidative type; fibers containing slow myosin isoforms) and in some type IIA fibers (fast-twitch oxidative type) in pigs¹⁹ and cattle¹¹⁶, and lipid content is related to the shear force of the meat²⁰. Histochemical myofiber classification techniques have been used to study the postnatal changes in muscle properties^{13,117,120}, the effects of diet on muscle characteristics^{14,56}, differences in myofiber properties in breeds^{32,64}, and the effects of obesity^{66,71}, hormones and drugs^{92,97,98}. In pigs, muscle fiber type influences heat-induced aggregation of porcine salt-soluble protein, and therefore different processing treatments should be used based on fiber type for optimum quality of meat products¹⁰⁷. In Japanese Black cattle, the composition of muscle fiber types is related to marbling score^{24-26,48,52}, which is the most important parameter of meat quality in Japan. The muscle fiber type distribution can be altered by diet in growing calves⁵⁶ and also by

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excise in sheep¹³³). Thus, the study of the physiological properties of muscle fibers plays an important role in breeding and feeding of meat-producing domestic animals.

Muscle fibers are not static structures but represent versatile entities which adapt to altered functional demands, hormonal signals and changes in neural input^{99,100}). Their dynamic nature makes it difficult to categorize them into distinct units. Therefore, applying any type of rigid structural and functional confines results in an oversimplification which does not take into account the plasticity of phenotypic expression. However, histochemical myofiber typing is an extremely useful tool in many fields of muscle physiology. Classification schemes have helped to define the functional, metabolic and molecular properties of muscle fibers selected on the basis of their histochemical appearance. Under such conditions, certain muscle fiber populations with similar functional properties and molecular profiles may be delineated, *e.g.* fast- and slow-twitch myofibers, and oxidative and glycolytic myofibers^{99,100}). Major myofiber types, *e.g.* type I, type IIA and type IIB, represent large groups of muscle fibers displaying similar phenotypes (Table 1). Such groups reflect stable programs or stages of gene expression with preferential combinations of sarcoplasmic and myofibrillar protein isoforms^{6,16-18,28-31,101,106,108,134,135}), for example fast- and slow-myosins. More-

over, they are the direct result of predominant patterns of functional demand and neuromuscular activity¹⁰⁰). Myofibers within a particular group may show similar structural and functional properties, but the most similar muscle fibers will be those within the same motor unit. Theoretically, there will be at least as many fiber types as there are motor units in a muscle. Within a muscle, a myofiber type may exhibit a continuum of structural and functional properties which overlap with those of other fiber types. As increasing numbers of different muscles and species are investigated and more refined high-resolution techniques are developed and applied, the full extent of cellular and molecular diversity of skeletal muscle should become evident. Histochemical myofiber classification schemes depend upon subjective evaluation and are limited by the restricted resolution of the methods applied^{10,11,13,55,99,100,120}). Only a small number of major myofiber types can be discriminated by qualitative histochemical methods. Nevertheless, histochemical myofiber typing has proven useful in numerous studies concerned with structure function relationships in normal and pathological muscles¹⁰⁰).

Classically, two different enzyme-histochemical approaches allow for the separation of myofiber types^{10,11,13,55,99,100,120}). (1) One method is based upon myofibrillar myosin adenosine

Table 1. Histochemical characteristics of myofiber types in the skeletal muscles of farm animals

Myofiber types	Anti-fast-myosin antibody	Acid-stable myosin ATPase ^a	Alkali-stable myosin ATPase ^b	SDH ^c	NADH-TR ^d	PhR ^e
I	Negative	++++ ^f	—	++++	++++	—/+
IIA	Positive	—	++++	++++	++++	+++
IIB	Positive	—	++++	+	+	++++

^a Preincubation at pH 4.3.

^b Preincubation at pH 10.5.

^c Succinate dehydrogenase.

^d Reduced nicotinamide adenine dinucleotide-tetrazolium reductase.

^e α -Glycogen phosphorylase.

^f Enzyme activities: —, unreactive; +, weak; +++, strong; +++++, very strong.

triphosphatase (ATPase) activity and the other upon reference enzymes of anaerobic and aerobic energy metabolism. The initial distinction of two myofibrillar myosin ATPase-based fiber types, *i.e.* type I and type II, was made possible by the use of a histochemical assay for ATPase activity (Table 1). Shortly thereafter, it was demonstrated that fast and slow skeletal muscles contain myosins differing in their specific actin-activated and Ca^{2+} -dependent ATPase activities and that this is correlated with differences in speed of contraction^{6,16-18,28,57,58,85,102}. It became clear that the histochemical differences in the activity of myosin ATPase between type I and type II fibers correspond to differences in contractile properties. Subsequently, differences in proteolytic fragments and antigenicity suggested the existence of myosin heavy chain isoforms¹⁰³. (2) Different types of muscle fibers have also been distinguished on the basis of histochemical reactions for enzymes of aerobic oxidative metabolism, *e.g.* succinate dehydrogenase (SDH), cytochrome oxidase, nicotinamide adenine dinucleotide (NADH) tetrazolium reductase, etc.¹⁰⁰. Two major myofiber types derived from differences in these enzyme activities basically reflect differences in mitochondrial content (high and low), and therefore are primarily related to differences in aerobic oxidative potential. Myofiber types can also be delineated histochemically by the inverse relationship between the activity of glycolytic metabolic enzymes, *e.g.* glycogen phosphorylase, mitochondrial glycerophosphate dehydrogenase, etc., and the activities of the enzymes involved in aerobic oxidative metabolism. As described above, this conventional histochemical classification of myofibers have been used for qualitative analysis of the physiological properties of skeletal muscles in farm animals.

Histomorphometrical analysis of skeletal muscles

Histomorphometry is a conventional quanti-

tative method for assessment of skeletal muscle properties^{12,15,76,78,81,85}. As described above, the physiological characteristics of mammalian skeletal muscles have been assessed by histochemical and biochemical methods. The muscle fibers can be classified into three major myofiber types, and then further into some minor types according to the intensity of enzyme activity demonstrated histochemically in individual myofibers. The cross sectional area of each myofiber type in muscle samples was measured by morphometrical techniques using a computer-aided automatic image analyzer^{12,15,76,78,81,85}. The Division of Computer Research and Technology of the National Institute of Health (NIH), in USA has made the image processing application NIH Image available without charge. NIH Image is available for the Macintosh and also for IBM - compatible computers running Windows. NIH Image can be downloaded by anonymous ftp from <ftp://zippy.nih.gov/pub/nih-image/>. Additional programs, macros, plug-ins, complete source code, etc., are available from the NIH Web site (<http://rsb.info.nih.gov/nih-image/>). Detailed questions regarding the application will be answered by Dr. Mark Vivino (mvivino@helix.nih.gov). NIH Image supports many standard image processing functions including contrast enhancement, density profiling, smoothing, sharpening, edge detection, median filtering and spatial convolution with user defined kernels. It can be used to measure area, mean, centroid, perimeter, etc. of user-defined regions of interest, and also performs automated analysis. As image processing and analysis methods differ among different programs, it is difficult to compare image analysis data presented by different research groups. If all investigators used the same image processing and analysis program, *e.g.* NIH Image, the image data from different laboratories would be compatible with each other. An example of image analysis of goat myofibers performed

using NIH Image is shown in Fig. 1. NIH Image running on a Power Macintosh 9600/OS 8.0 computer with 672 MB of RAM was used to perform automated analysis. As reported previously^{76,78,81,85}, frozen serial sections of goat skeletal muscles were incubated for enzyme histochemical analysis of myosin ATPase, succinate dehydrogenase, NADH tetrazolium reductase and α -glycogen phosphorylase activities, and stained immunohistochemically using a monoclonal antibody against fast-myosin. Serial sections stained for SDH and fast-myosin were photographed, and then these photographs were scanned into a Power Macintosh computer (Fig. 1A and B, respectively) using a film scanner. Histochemical reactions were automatically divided into two classes, *i.e.* negative and positive (binarization) (Fig. 1C and D, respectively), using NIH Image on a Power Macintosh computer. Myofibers (at least 500 fibers/section; three sections/muscle) were classified into three major types according to differences in reactivity for the enzymes and with the antibody (Fig. 1E). Species-specific myofiber types were reported in the skeletal muscles of ruminants¹⁰⁹⁻¹¹¹. Type Ib fibers which showed weak SDH activity and were negative for fast-myosin were observed in the goat skeletal muscles and were categorized as type I myofibers for simplicity⁸⁵. The numbers of fibers of each type and the transverse area of each myofiber in the sections were automatically measured using the image analysis system (Table 2), and then the proportions of each myofiber type were calculated (Table 3)^{76,78,81,85}.

Immunohistochemical quantitative analysis of skeletal muscles

Conventional histomorphometrical techniques preserve the muscle structure and require very small samples (thin sections mounted on glass slides), but they cannot provide information regarding the precise quantitative properties of the skeletal muscles^{76,78,81,85}. Even with use of a automated image analysis

system as described above, analysis of the muscle properties still requires in the use of histochemical techniques and is time-consuming. The pyrophosphoric acid polyacrylamide gel electrophoresis method^{16-18,76,78,81,85} allows precise quantitative evaluation but requires large tissue samples and the muscle structure is destroyed because samples must be homogenized. For example, electrophoresis has been used to analyze myosin isoforms, the major contractile components which determine the contractile properties of muscle fibers²⁸, and at least 100 mg of wet tissue was required^{76,78,81,85}. Myosin is an asymmetrical, hexameric protein consisting of four light chains (approximately 20 kDa each) and two heavy chains (approximately 200 kDa each)^{6,16-18,28-31,57,58,106,134,135}. This ubiquitous protein, found in both muscle and nonmuscle cells, makes up the largest part of the contractile apparatus in skeletal muscle fibers. The two myosin heavy chains intertwine at the carboxyl terminus forming an α -helical coiled coil (the rod part). The amino terminus of each heavy chain forms a globular head region such that each holomyosin contains two heads. The rod is responsible for the aggregation of myosin molecules into bipolar thick filaments, whereas the head region is the site of ATPase activity and actin binding. Myofibers contain at least two types of myosin. Fast-myosin is the major contractile component that determines the contractile properties of muscle fibers, and thus is a key protein for their precise evaluation. Fast-myosin content in the skeletal muscles have been measured by conventional biochemical electrophoretic methods. Recently, Picard *et al.*¹⁰² reported the use of an enzyme-linked immunosorbent assay (ELISA) with a monoclonal antibody specific for type I myosin heavy chain (slow-myosin) to quantitatively assess the contractile properties of bovine skeletal muscles. Their biochemical ELISA method was sensitive sufficiently for precise measurement and was a simple procedure compared with conventional

Fig. 1. Light micrographs showing serial cross-sections from a horizontal slice of the tensor fasciae latae muscle of the goat (A and B). The sections were stained enzyme histochemically for succinate dehydrogenase (SDH) (A). Strong staining was observed in type I and IIA fibers, moderate staining was seen in type Ib, and weak staining was demonstrated in type IIB myofibers. After immunohistochemical measurement of fast-myosin, an additional procedure for immunohistochemical staining of fast-myosin was applied to the serial sections (B). Positive staining was observed in type II fibers, but no staining was seen in type I fibers. These photographs were scanned into a computer, and then the histochemical reactions were automatically divided into negative and positive reactions using NIH Image (C and D, respectively). These images were automatically combined, and then myofibers were classified into three major myofiber types (type I, IIA and IIB) according to differences in reactivity for the enzyme and with the antibody (E). In goat muscles, species-specific type Ib myofibers with moderate SDH activity were observed, and they were finally classified as type I.

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Table 2. Transverse area of myofiber types in the mid-belly portion of goat skeletal muscles

Muscle	Type I fibers (μm^2)	Type IIA fibers (μm^2)	Type IIB fibers (μm^2)
Muscles of the upper forelimb			
Latissimus dorsi	1,943 $\pm$ 169 ^a	4,234 $\pm$ 266 ^a	7,363 $\pm$ 368 ^a
Superficial pectoralis	2,101 $\pm$ 232 ^a	3,120 $\pm$ 282 ^b	5,997 $\pm$ 357 ^b
Deep pectoralis	2,413 $\pm$ 185 ^b	3,326 $\pm$ 321 ^b	6,011 $\pm$ 302 ^b
Tensor faciae antebrachii	2,048 $\pm$ 199 ^a	4,254 $\pm$ 282 ^a	7,126 $\pm$ 380 ^a
Cleidobrachialis	1,857 $\pm$ 201 ^a	3,940 $\pm$ 336 ^a	6,557 $\pm$ 522 ^{ab}
Triceps brachii, lateral head	2,014 $\pm$ 201 ^a	4,085 $\pm$ 302 ^a	7,451 $\pm$ 874 ^a
Triceps brachii, medial head	1,662 $\pm$ 204 ^c	3,684 $\pm$ 331 ^{ac}	6,358 $\pm$ 481 ^b
Triceps brachii, long head	1,984 $\pm$ 331 ^a	4,014 $\pm$ 332 ^a	7,205 $\pm$ 484 ^a
Biceps brachii	2,788 $\pm$ 303 ^d	3,308 $\pm$ 299 ^b	6,047 $\pm$ 441 ^b
Brachialis	3,054 $\pm$ 387 ^d	3,406 $\pm$ 526 ^{bc}	5,870 $\pm$ 558 ^b
Muscles of the lower forelimb			
Extensor carpi radialis	1,894 $\pm$ 158 ^a	4,520 $\pm$ 322 ^d	7,455 $\pm$ 405 ^a
Flexor carpi radialis	2,524 $\pm$ 252 ^{bd}	3,478 $\pm$ 362 ^{bc}	6,325 $\pm$ 236 ^b
Extensor carpi ulnaris	1,944 $\pm$ 221 ^a	4,418 $\pm$ 298 ^{ad}	7,244 $\pm$ 581 ^a
Flexor carpi ulnaris	1,887 $\pm$ 220 ^a	4,218 $\pm$ 295 ^a	7,401 $\pm$ 344 ^a
Deep digital flexor	2,210 $\pm$ 205 ^a	3,655 $\pm$ 322 ^{ac}	6,940 $\pm$ 350 ^a
Superficial digital flexor	2,754 $\pm$ 381 ^d	3,401 $\pm$ 281 ^{bc}	6,335 $\pm$ 422 ^b
Common digital extensor	2,984 $\pm$ 415 ^d	3,321 $\pm$ 365 ^b	5,968 $\pm$ 584 ^b
Lateral digital extensor	2,018 $\pm$ 254 ^a	3,804 $\pm$ 409 ^a	6,875 $\pm$ 560 ^a
Pronator teres	2,897 $\pm$ 336 ^d	3,059 $\pm$ 387 ^b	5,940 $\pm$ 491 ^b
Adductor pollicis longus	3,011 $\pm$ 425 ^d	3,266 $\pm$ 369 ^b	6,387 $\pm$ 522 ^b
Muscles of the upper hindlimb			
Sartorius	2,144 $\pm$ 224 ^a	4,519 $\pm$ 295 ^d	7,244 $\pm$ 422 ^a
Biceps femoris	2,201 $\pm$ 276 ^a	4,908 $\pm$ 462 ^c	6,669 $\pm$ 438 ^a
Rectus femoris	2,011 $\pm$ 322 ^a	4,885 $\pm$ 351 ^a	7,544 $\pm$ 520 ^a
Vastus lateralis	1,957 $\pm$ 306 ^a	4,598 $\pm$ 435 ^d	6,977 $\pm$ 455 ^a
Vastus medialis	2,044 $\pm$ 269 ^a	4,925 $\pm$ 406 ^c	7,411 $\pm$ 426 ^a
Vastus intermedialis	3,804 $\pm$ 334 ^e	3,994 $\pm$ 501 ^a	6,097 $\pm$ 328 ^b
Adductores	2,620 $\pm$ 318 ^{bd}	3,694 $\pm$ 336 ^{ac}	6,588 $\pm$ 362 ^{ab}
Semitendinosus	1,987 $\pm$ 199 ^a	4,075 $\pm$ 433 ^a	6,336 $\pm$ 457 ^d
Semimembranosus	2,158 $\pm$ 306 ^a	3,997 $\pm$ 336 ^a	6,654 $\pm$ 380 ^{ab}
Pectineus	3,011 $\pm$ 247 ^d	4,087 $\pm$ 462 ^a	5,877 $\pm$ 531 ^b
Gracilis	2,698 $\pm$ 305 ^d	4,478 $\pm$ 455 ^{ad}	5,960 $\pm$ 526 ^b
Muscles of the lower hindlimb			
Gastrocnemius, lateral head	2,403 $\pm$ 131 ^b	3,460 $\pm$ 409 ^{bc}	4,392 $\pm$ 368 ^c
Gastrocnemius, medial head	1,874 $\pm$ 234 ^a	3,788 $\pm$ 351 ^{ac}	5,966 $\pm$ 411 ^b
Soleus	2,855 $\pm$ 406 ^d	3,604 $\pm$ 350 ^{ac}	3,969 $\pm$ 422 ^c
Cranial tibialis	2,622 $\pm$ 252 ^{bd}	3,478 $\pm$ 362 ^{bc}	6,325 $\pm$ 236 ^b
Long digital extensor	1,955 $\pm$ 321 ^a	4,155 $\pm$ 419 ^a	6,325 $\pm$ 408 ^b
Lateral digital extensor	3,014 $\pm$ 441 ^d	3,965 $\pm$ 501 ^a	4,115 $\pm$ 521 ^c
Peroneus longus	2,877 $\pm$ 258 ^d	3,448 $\pm$ 342 ^{bc}	5,988 $\pm$ 479 ^b
Deep digital flexor	2,322 $\pm$ 252 ^{ab}	3,478 $\pm$ 362 ^{bc}	6,058 $\pm$ 236 ^b
Superficial digital flexor	2,031 $\pm$ 155 ^a	3,684 $\pm$ 461 ^{ac}	6,448 $\pm$ 363 ^b
Popliteus	2,541 $\pm$ 332 ^{bd}	3,804 $\pm$ 455 ^a	5,840 $\pm$ 462 ^a

a, b, c, d, e : Means followed by different superscripts differed significantly ($P < 0.05$).

Quantification of Muscle Properties

Table 3. Populations of myofiber types in the mid-belly portion of goat skeletal muscles

Muscle	Type I fibers (fibers/mm ²)	Type IIA fibers (fibers/mm ²)	Type IIB fibers (fibers/mm ²)
Muscles of the upper forelimb			
Latissimus dorsi	515 ± 26 ^a	236 ± 22 ^a	136 ± 43 ^a
Superficial pectoralis	476 ± 24 ^a	321 ± 19 ^b	167 ± 33 ^a
Deep pectoralis	414 ± 33 ^b	301 ± 39 ^b	166 ± 33 ^a
Tensor faciae antebrachii	488 ± 14 ^a	235 ± 18 ^a	140 ± 39 ^a
Cleidobrachialis	539 ± 16 ^a	254 ± 16 ^a	153 ± 21 ^a
Triceps brachii, lateral head	497 ± 20 ^a	245 ± 17 ^a	134 ± 30 ^a
Triceps brachii, medial head	602 ± 67 ^c	271 ± 29 ^{ab}	157 ± 29 ^a
Triceps brachii, long head	504 ± 24 ^a	249 ± 32 ^a	139 ± 36 ^a
Biceps brachii	359 ± 57 ^d	302 ± 25 ^b	165 ± 25 ^a
Brachialis	327 ± 31 ^d	294 ± 26 ^b	170 ± 26 ^a
Muscles of the lower forelimb			
Extensor carpi radialis	528 ± 19 ^a	221 ± 31 ^a	134 ± 41 ^a
Flexor carpi radialis	396 ± 36 ^b	288 ± 42 ^b	158 ± 24 ^a
Extensor carpi ulnaris	514 ± 15 ^a	226 ± 31 ^a	138 ± 34 ^a
Flexor carpi ulnaris	530 ± 31 ^a	237 ± 19 ^a	135 ± 33 ^a
Deep digital flexor	452 ± 46 ^{ab}	274 ± 41 ^{ab}	144 ± 31 ^a
Superficial digital flexor	363 ± 57 ^{bd}	294 ± 15 ^b	158 ± 25 ^a
Common digital extensor	335 ± 22 ^d	301 ± 28 ^b	168 ± 27 ^a
Lateral digital extensor	496 ± 19 ^a	263 ± 34 ^{ab}	145 ± 24 ^a
Pronator teres	345 ± 30 ^d	327 ± 36 ^b	168 ± 24 ^a
Adductor pollicis longus	332 ± 15 ^d	306 ± 20 ^b	157 ± 20 ^a
Muscles of the upper hindlimb			
Sartorius	466 ± 21 ^{ab}	221 ± 18 ^a	138 ± 35 ^a
Biceps femoris	454 ± 25 ^{ab}	204 ± 22 ^a	150 ± 30 ^a
Rectus femoris	497 ± 14 ^a	205 ± 14 ^a	133 ± 28 ^a
Vastus lateralis	511 ± 18 ^a	217 ± 29 ^a	143 ± 33 ^a
Vastus medialis	489 ± 23 ^a	203 ± 35 ^a	135 ± 27 ^a
Vastus intermedialis	263 ± 45 ^c	250 ± 30 ^{ab}	164 ± 15 ^a
Adductores	382 ± 44 ^{bd}	271 ± 38 ^{ab}	152 ± 27 ^a
Semitendinosus	503 ± 16 ^a	245 ± 20 ^a	158 ± 36 ^a
Semimembranosus	463 ± 8 ^{ab}	250 ± 43 ^{ab}	150 ± 25 ^a
Pectineus	332 ± 37 ^d	245 ± 22 ^a	170 ± 21 ^a
Gracilis	371 ± 23 ^{bd}	223 ± 32 ^a	168 ± 9 ^a
Muscles of the lower hindlimb			
Gastrocnemius, lateral head	416 ± 21 ^b	289 ± 26 ^b	228 ± 27 ^b
Gastrocnemius, medial head	534 ± 22 ^a	264 ± 37 ^{ab}	168 ± 22 ^a
Soleus	350 ± 60 ^{bd}	277 ± 36 ^{ab}	252 ± 20 ^b
Cranial tibialis	381 ± 40 ^{bd}	288 ± 16 ^{ab}	158 ± 29 ^a
Long digital extensor	512 ± 16 ^a	241 ± 19 ^a	158 ± 33 ^a
Lateral digital extensor	332 ± 47 ^d	252 ± 32 ^{ab}	243 ± 18 ^b
Peroneus longus	348 ± 27 ^d	290 ± 28 ^b	167 ± 33 ^a
Deep digital flexor	431 ± 46 ^b	288 ± 24 ^b	165 ± 43 ^a
Superficial digital flexor	492 ± 17 ^a	271 ± 32 ^{ab}	155 ± 36 ^a
Popliteus	394 ± 27 ^b	263 ± 38 ^{ab}	171 ± 31 ^a

a, b, c, d, e : Means followed by different superscripts differed significantly (P < 0.05).

electrophoresis techniques, but the tissue structure was also completely destroyed. A new sensitive method for quantitative analysis in histological muscle sections, in which the structure can be well-preserved, is needed for quantitative evaluation of the properties of skeletal muscles.

We have developed a novel method for quantification of fast-myosin, which is a key protein for precise evaluation of the contractile properties of skeletal muscle, in sections mounted on glass slides^{76, 78, 80, 81, 85}. A flow diagram of

this histochemical micromethod is shown in Fig. 2. Frozen muscle sections mounted on glass slides are used in this technique as analogues of the antigen-precoated wells of ELISA plates. Using frozen sections of the masseter and gastrocnemius muscles from five mammals (adult Holstein-Friesian cows, female Berkshire pigs, female Saanen goats, male Sprague-Dawley rats and female Japanese White rabbits), we demonstrated the usefulness of our immunohistochemical method for the quantification of fast-myosin

I. Preparation of direct standard curve

1. Coat the wells of the ELISA plate with purified fast-myosin standard.
2. Incubate with first antibody (mouse anti-rabbit skeletal fast-myosin monoclonal IgG antibody; 1/400 dilution) (4 °C, 18 h).
3. Transfer the supernatant to ELISA plate precoated with fast-myosin (continue on II-2).
4. Rinse (x5, 5 min each), and then incubate with second antibody (horseradish peroxidase-conjugated goat anti-mouse IgG antibody; 1/400 dilution) (37 °C, 2 h).
5. Rinse (x5, 5 min each), and then incubate with chromogenic substrate solution (PBS containing 3 mM 4 aminoantipyrine, 30 mM phenol and 2 mM H₂O₂) (37 °C, 1 h).
8. Stop the reaction with 1 M NaN₃ solution, and then determine the OD at 450 nm.

II. Preparation of indirect standard curve

1. Incubate fast-myosin standard solution with first antibody.
2. Transfer the supernatant of I-2 (continue from I-2) and II-1 to ELISA plate precoated with fast-myosin.
3. Incubate, rinse and then incubate with second antibody.
4. Rinse, and then incubate with chromogenic substrate solution.
5. Stop the reaction, and then determine the OD at 450 nm.

III. Measurement of fast-myosin content in sections

1. Preincubate with normal goat serum, rinse and then incubate with first antibody.
2. Rinse and inhibit the endogenous peroxidase activity.
3. Rinse, and then incubate with second antibody.
4. Rinse, and then incubate with chromogenic substrate solution.
5. Stop the reaction, and then determine the OD at 450 nm (continue on IV-1).

IV. Measurement of total protein content in sections

1. Remove the antigen-antibody complex.
 2. Rinse and stain with sirius red F3BA and fast green FCF.
 3. Rinse, extract colors and then read at 540 and 630 nm.
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Fig. 2. Flow diagram of immunohistochemical measurement of fast-myosin in skeletal muscle sections.

responsible for the contractile properties of muscle fibers⁷⁹). Then, we examined the contents of fast-myosin in different portions of the limb muscles of female Saanen goats to determine the usefulness of this micromethod for evaluating the physiological properties of muscle and meat quality (Table 4)^{76,81}). This immunohistochemical method based on the principle of the ELISA technique requires only small frozen sections for precise quantification, and the tissue structure is completely preserved. This preservation is important for precise evaluation of the physiological properties of skeletal muscles. Furthermore, even after measurement, the localization of fast-myosin in the tissue sections can be visualized by additional immunohistochemical staining (Fig. 1B). We have developed double measurement method by which fast-myosin and type I collagen levels can be assessed on the same sections (in preparation). A more complete analysis of fast-myosin isoform contents in the skeletal muscles from domestic animals may lead to a better understanding of their physiological properties and meat quality.

On immunohistochemical micromethod, based on the principle of the ELISA technique, requires only one frozen section mounted on a glass slide for precise quantification, and the tissue structure is completely preserved. This method is sufficiently sensitive for estimation of the quantity of fast-myosin isoforms using frozen tissue sections. Conventionally, the intensity of immunohistochemical staining has been evaluated by subjective methods, for example by scoring. However, histological scoring is not quantitative, and the results of such scoring differ among different observers. The intensity of immunoreactivity of a target substance in frozen tissue sections can be quantified by our immunohistochemical micromethod. This novel micromethod can be used for measurement of other proteins, e.g. extracellular matrix proteins, actin, myoglobin,

Table 4. Fast-myosin level assessed by immunohistochemical method in the mid-belly portion of goat skeletal muscles

Muscle	Fast-myosin (mg/g of total protein)
Muscles of the upper forelimb	
Latissimus dorsi	178±18 ^a
Superficial pectoralis	169±16 ^a
Deep pectoralis	145±12 ^b
Tensor faciae antebrachii	182±21 ^a
Cleidobrachialis	194±19 ^a
Triceps brachii, lateral head	205±18 ^a
Triceps brachii, medial head	15±4 ^c
Triceps brachii, long head	189±14 ^a
Biceps brachii	51±6 ^d
Brachialis	87±12 ^c
Muscles of the lower forelimb	
Extensor carpi radialis	186±12 ^a
Flexor carpi radialis	141±15 ^b
Extensor carpi ulnaris	192±12 ^a
Flexor carpi ulnaris	174±15 ^a
Deep digital flexor	152±10 ^b
Superficial digital flexor	41±6 ^d
Common digital extensor	148±13 ^b
Lateral digital extensor	142±17 ^b
Pronator teres	128±12 ^{be}
Adductor pollicis longus	145±17 ^b
Muscles of the upper hindlimb	
Sartorius	197±13 ^a
Biceps femoris	193±16 ^a
Rectus femoris	201±14 ^a
Vastus lateralis	208±21 ^a
Vastus medialis	184±11 ^a
Vastus intermedialis	24±8 ^c
Adductores	112±14 ^{be}
Semitendinosus	204±19 ^a
Semimembranosus	212±18 ^a
Pectineus	148±15 ^b
Gracilis	160±18 ^a
Muscles of the lower hindlimb	
Gastrocnemius, lateral head	205±10 ^a
Gastrocnemius, medial head	184±16 ^a
Soleus	19±7 ^c
Cranial tibialis	130±14 ^b
Long digital extensor	201±21 ^a
Lateral digital extensor	74±9 ^e
Peroneus longus	144±11 ^b
Deep digital flexor	136±17 ^b
Superficial digital flexor	191±16 ^a
Popliteus	105±7 ^{be}

^{a,b,c,d,e}: Means followed by different superscripts differed significantly ($P < 0.05$).

calpain, etc., in histological muscle sections. Thus, this quantitative technique could be used to study the physiological characteristics of muscle that determine meat quality. Furthermore, this technique is available for quantitative analysis of other organs under physiological and pathological conditions. Our previous studies revealed that this immunohistochemical microquantification method is also a useful tool for quantitative histopathological diagnosis in patients and / or experimental animals with hepatic^{28, 69, 70, 73, 79, 80, 84, 86-88)}, renal^{75, 79, 90)} and cutaneous⁹¹⁾ failure.

Noninvasive Quantitative Assessment of Muscle Energy Metabolism and Catabolism by ^{31}P Nuclear Magnetic Resonance Spectroscopy

We have developed a ^{31}P nuclear magnetic resonance (^{31}P NMR) spectroscopy method for *in vivo* measurement of hepatic adenosine triphosphate (ATP) level^{72, 74, 83)}. This *in vivo* ^{31}P NMR technique is sufficiently sensitive to predict liver damage induced by environmental toxic chemicals^{23, 74)} or by ischemic damage^{23, 89)}, and to evaluate the improvement of liver function by synthetic drugs and natural medicines⁸³⁾. Recently, we modified this structure biological method for assessment of muscle energy metabolism and catabolism^{4, 5)}. The process of catabolism in postmortem muscle has been intensively investigated by conventional biochemical methods. However, the destructive nature of these conventional methods inevitably induces the breakdown of high-energy phosphates. Our ^{31}P NMR spectroscopy technique is a useful and powerful tool for investigating tissue metabolism in a noninvasive manner^{4, 5)}. The NMR method enables us to simultaneously estimate the levels of high-energy phosphates (ATP, adenosine diphosphate : ADP, adenosine monophosphate, etc.), creatine phosphate, inorganic phosphate, sugar phosphates, etc., in a single specimen.

This NMR method also provides information about intracellular pH^{4, 5)}. The detailed methodology was confirmed using muscle samples from laboratory animals⁵⁾. In our previous study⁵⁾, ^{31}P NMR was used to investigate the catabolism of phosphorylated metabolites and the intracellular pH changes in isolated soleus and extensor digitorum longus (EDL) muscles of rats. These two muscles are typical slow-twitch (type I myofiber-dominant muscle) and fast-twitch muscles (type II myofiber-dominant muscle), respectively. Both muscles can be dissected out without cutting their myofibers. Although there have been several NMR studies of the relationship between histochemical myofiber type composition and postmortem catabolism, none used muscle specimens that were undamaged during sample preparation or measured high-energy phosphate levels. We estimated the time course of catabolism of phosphorylated metabolites in undamaged rat muscles by means of our noninvasive ^{31}P NMR method. The soleus and the EDL muscles were obtained without any muscle damage. *In vivo* ^{31}P NMR spectra of these muscles were acquired using a surface coil, and continuous changes in muscle energy metabolism were quantified (Fig. 3). Briefly, each animal was anesthetized by intraperitoneal injection of sodium pentobarbital. During the NMR examination, the animal was maintained in a probe-chamber at a controlled air temperature of 25°C. ^{31}P NMR spectra were obtained using a wide bore type NMR spectrometer (JNM α -400, JEOL, Tokyo, Japan) equipped with a superconducting magnet (9.20 Tesla ; Oxford Instruments, Bedford, MA, USA). The animal was mounted on a custom made surface coil probe (two-turns, 2.0 cm diameter) which was tuned to ^{31}P at 161.7 MHz and used in conjunction with a ferrite screen composed of strips of computer-tape (Scotch 700 ; Three M Co., St. Louis, MO) to eliminate ^{31}P NMR signals arising from cutaneous tissues. Field homogeneity was

Fig. 3. A representative *in vivo* ^{31}P NMR spectrum of rat extensor digitorum longus muscle (fast-twitch muscle or type II myofiber-dominant muscle). Prominent peaks observed were sugar phosphates, inorganic phosphate, creatine phosphate, nucleotide triphosphates (mainly γ -ATP, α -ATP, and β -ATP). The γ -ATP and α -ATP signals contained contributions from β - and α -ADP, respectively. The signal corresponding to β -ATP, which has no overlap with other phosphate signals, was used to quantify ATP.

controlled by adjusting shim parameters using the water ^1H signal derived from the muscle tissue sample. Acquisition conditions were set as follows to obtain fully relaxed signals: pulse width, 20 μsec ; pulse repetition time, 3.0 s; 200 scans were accumulated. Thus, it took 10 min for each measurement. After exponential multiplication, the accumulated free induction decays were subjected to Fourier transformation. Individual spectral peak areas were calculated by computer integration with JEOL software, and the creatine phosphate peak was used as a positional reference (0 ppm). The relative amount of each compound was converted to absolute concentration by comparison with the data from an external reference (20 mM methylene diphosphonate in water) in a spherical bulb. The relative ATP levels were obtained by taking the ratio of the

β -phosphate peak of ATP (β -ATP) to the reference peak, and then the β -ATP/inorganic phosphate peak area ratio (β -ATP/ Pi ratio) was calculated to evaluate *in vivo* energy metabolism. Beta - ATP / inorganic phosphate ratio represents the high energy state⁷⁾. The intracellular pH was determined from the chemical shift difference between the Pi peak and the α -phosphate peak of ATP⁷⁾.

In both muscles, rapid depletion of creatine phosphate was followed by a decrease in ATP. These high-energy phosphates disappeared earlier in the soleus than in the EDL. In both muscles, inorganic phosphate showed a marked increase in a biphasic pattern, and sugar phosphate levels also showed significant increases. The NMR-visible total phosphates increased significantly in the soleus during the latter stage of observation. This increase in NMR-visible total phosphates was attributed to an increase in inorganic phosphate. Although the two muscles showed the same initial and final intracellular pH (7.21 and 5.90, respectively), the pattern of intracellular pH decrease differed between these muscles. The rapid decrease in intracellular pH in the soleus in the early stage suggested that non-lactic acid acidosis (nonlactic acidosis) plays a significant role in postmortem changes. Although the level of NMR-visible total phosphates during catabolism increased only in the soleus muscle, it is not clear whether this observation was attributable to the use of intact muscles with no myofiber damage. The rapid intracellular pH decrease observed in the soleus muscle in the early stage cannot be explained by the established theory that the postmortem pH decrease is caused by lactate formation due to glycolysis. These findings indicated that nonlactic acidosis plays a significant role in the postmortem pH change. Ravalec *et al.*¹⁰⁵⁾ demonstrated that the improvement of muscular oxidative capacity by training is associated with slight acidosis and ATP depletion in exercising muscles by the *in vivo* ^{31}P NMR

technique. Metabolic and mechanical properties of the skeletal muscles of female rats subjected to endurance training on a treadmill were studied by *in vivo* multistep fatigue test, and ^{31}P NMR was used to follow energy metabolism and pH. The oxidative capacity of the skeletal muscles was evaluated from the relationship between ADP calculated from the creatine kinase equilibrium and work and from the rate of phosphocreatine resynthesis following exercise. In trained muscles, ADP production was lower per unit of mechanical performance, showing an improvement of oxidative metabolism. However, the phosphocreatine resynthesis rate was not modified. Slight acidosis and ATP depletion were observed from the beginning of the fatigue test. These modifications suggested changes of the creatine kinase equilibrium favoring mitochondrial ATP production.

In farm animals, investigation of the time course of the catabolism of high energy phosphates in the skeletal muscle is very important to evaluate meat quality⁴. We used female Saanen goats as a model of ruminants and evaluated the goat supraspinatus (slow-twitch muscle: Type I fiber-dominant, representative oxidative energy producing muscle) and tensor fasciae latae (fast-twitch muscle: Type IIB fiber-dominant, representative glycolytic energy producing muscle) muscles using non-destructive ^{31}P NMR spectroscopy. In both muscles, rapid depletion of creatine phosphate was followed by a decrease in ATP. These high energy phosphates disappeared earlier in the supraspinatus than in the tensor fasciae latae. Glycerophosphorylcholine was detected only in the goat supraspinatus muscle. Intracellular pH decreased faster in the supraspinatus than in the tensor fasciae latae, although the two muscles had the same final intracellular pH. Our findings confirmed that the physiological characteristics of skeletal muscles such as histochemical myofiber type composition or contractile capacities are close-

ly related to meat quality, and that type II myofiber-dominant muscles mature more quickly than those dominated by type I myofibers. In both experimental and domestic animals, a rapid decrease in intracellular pH of type I myofiber-dominant muscles was confirmed^{4,5}. In porcine and bovine skeletal muscles, the ^{31}P NMR technique is also useful to assess postmortem metabolism and evaluate meat quality¹³⁶. These findings indicated that the ^{31}P NMR method could be used to quantitatively evaluate the muscle energy metabolic characteristics that determine meat quality of domestic animals. Moreover, Geers *et al.*²² used the NMR method to detect halothane-sensitive genes in pigs. They estimated the energy metabolism of skeletal muscle tissue of young pigs (12–18 kg) using *in vivo* ^{31}P NMR spectroscopy. Their results, if confirmed with a large number of pigs, might provide new possibilities for selection procedures for leanness because a shift between genotypic and phenotypic variability with respect to halothane susceptibility was observed. Iotti *et al.*³⁵ used ^{31}P NMR spectroscopy to study *in vivo* the transport of inorganic phosphate from the cytosol into mitochondria as assessed by the kinetics of inorganic phosphate recovery during ischemia after aerobic exercise. Their results showed that inorganic phosphate transport from the cytosol into mitochondria is active in the absence of ATP biosynthesis and lasts for 30 seconds, possibly as a consequence of the decreased pH gradient due to transport of inorganic phosphate and H^+ associated with an inactive electron transport chain during ischemia. Radda¹⁰⁴ used the ^{31}P NMR method to quantitatively evaluate the capacity of energy metabolism in the skeletal muscles, and demonstrated that skeletal muscle energetics can be studied noninvasively at rest, during exercise and in recovery using ^{31}P NMR. In resting muscle, inorganic phosphate and total cellular phosphate concentration are regulated by Na^+ -dependent inorganic phosphate

transport. Insulin was shown to stimulate inorganic phosphate uptake in cultured G-8 muscle cells, in isolated rat soleus muscle, and in human muscle *in vivo*. Recovery after exercise is entirely oxidative; the rate of ATP synthesis is largely controlled by ADP, the concentration of which is determined by the creatine kinase equilibrium that includes the concentration of H^+ . At the onset of aerobic dynamic exercise, ATP is maintained largely by glycolysis, producing lactic acid, and by phosphocreatine breakdown. After vasodilation, ATP synthesis becomes predominantly oxidative. These ^{31}P NMR methods have been developed to observe muscle functions, *i.e.* contractile functions and energy metabolism, and recently 1H imaging NMR (magnetic resonance imaging: MRI) methods have been developed. MRI revealed not only the morphology of skeletal muscles but also their physiological functions. Ville *et al.*¹³⁷⁾ demonstrated that MRI is a useful tool to measure *in vivo* intramuscular fat content in porcine longissimus muscle. Yamaguchi *et al.*¹³⁸⁾ showed that the MRI technique is useful for evaluation of marbling of longissimus muscle of bovine carcasses.

In conclusion, the ^{31}P NMR method is very useful for evaluating the effects of exercise on skeletal muscle, for detecting pathogenic genes, and for determining the mechanisms of metabolic disorders. In farm animals, the ^{31}P NMR technique has been used for assessment of muscle physiological characteristics and meat quality^{4, 5, 83, 136)}. The MRI technique has also been applied for evaluation of meat quality of farm animals^{137, 138)}. Such structure-biological techniques will be useful for future analysis of the *in vivo* physiological properties of skeletal muscles.

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