3:14 The agreement of ATPase with immunology for typing myofibers of chicken skeletal muscle

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Introduction

In practice, myofiber types are commonly established by using histochemical reactions as indicators of contraction characteristics and energy metabolism. The reactions usually employed are for myofibrillar adenosine triphosphatase (ATPase) and for a mitochondrial enzyme such as succinic dehydrogenase (SDH). A massive literature about myofiber typing exists; it is difficult to interpret because of the variety of terminologies employed and because of the sensitivity of results to small alterations in procedure (e.g., preincubation pH for ATPase). One rationale for using the ATPase reaction is to provide an estimate of contractile properties of the myofiber. This has not been totally accepted, however. Guth (1973) concluded that the ATPase reaction cannot always be taken as indicative of the contraction speed of the myofiber, and Nemeth (1979) does not consider the reaction as always accurate for myofiber typing. Maxwell et al. (1982) reported that myosin ATPase activity, maximum shortening velocity and histochemical determination of myofiber type by ATPase do not always provide equivalent estimates of contractile properties, and they concluded that the histochemical procedures appeared to be the source of discrepancy.

A further complicating factor is that there are species differences for results obtained from the histochemical procedures. In addition, investigators have applied the nomenclature systems used for mammalian species (which contain only twitch myofibers) to avian species (which contain both twitch and tonic myofibers).

Immunochemical techniques have provided a new and specific approach to the problem of myofiber typing. Several recent papers report on investigations of slow and fast myofibers by using antibodies against different myosin isozymes (Arndt and Pepe, 1975; Billeter et al., 1980; Bormioli et al., 1980; Cantini et al., 1980; Gauthier, 1979; Gauthier and Lowey, 1977, 1979; Gauthier et al., 1982; Masaki, 1974; Snow et al., 1981).

We report in this manuscript an immunological procedure for typing fast and slow myofibers of chicken muscle based on specific antibodies to myosin heavy chain (MHC). The objectives were (1) to develop antibodies to MHC isozymes of fast and slow myofibers and establish their antigenic specificity by gel-electrophorisis-derived-enzyme linked-immusorbent-assay (GEDELISA), and (2) to compare the results of immunofluorescent study of myofiber types to the results from standard histochemical typing of myofibers.

Procedures

Preparation of Antibodies

Mature chickens were used throughout the study. Myofibrils prepared from the fast-twitch "white" region of chicken pectoralis (Gauthier and Lowey, 1977) or from chicken lower leg muscles were used as a source of fast-myofiber and mixed-myofiber (both fast and slow myofibers) MHC antigen respectively. The myofibrillar proteins were separated electrophoretically on polyacrylamide-SDS slab gels of 5% acrylamide - 0.07% Bis-acrylamide (Laemmli, 1970). Two milligrams of myofibrils were applied to a 3 mm thick slab gel, yielding a MHC band of approximately 0.8 mg.

The MHC band was prepared for immunization essentially by the procedure of Granger and Lazarides (1980). The MHC band was visualized by staining with Coomassie Blue followed by overnight destaining. The band was cut out with a scalpel, diced into small pieces, and equilibrated for 1-2 days in 0.1 M sodium phosphate, pH 7.4. The pieces were then homogenized in a motor driven glass/Teflon homogenizer along with a small amount of buffered saline solution (0.135 M NaCl - 10 mM potassium phosphate pH 7.2). The homogenate was emulsified using an equal volume of Freunds Complete adjuvant (Difco Co., Detroit, MI) by passing the mixture through a syringe several times. Incomplete adjuvant was used after the first injection.

Rabbits were injected subcutaneously at several sites along both sides of the spine on days 0, 14, 28, and 42 with 0.4 mg MHC. On day 50 each rabbit was bled (50 ml), and a crude immunoglobulin fraction was isolated from the serum by ammonium sulfate precipitation (25 g/100 ml). Subsequent bleedings were performed at six week intervals, with the rabbits receiving booster injections of 0.2 mg MHC 8 to 10 days before bleeding.

Affinity Purification of Antibodies

Two immunosorbent columns were prepared: one using fast-myofiber myosin isolated from the fast-myofiber region of chicken pectoralis and one using mixed-myofiber myosin from chicken leg muscles. Myosin was extracted using the methods of Nauss et al. 1969 and purified on DEAE-Sephadex (Offer et al., 1973). The myosin was coupled to cyanogen bromide activated Sepharose, at a concentration of 5 mg myosin/ml packed gel, by the procedure of March et al., 1974.

The crude immunoglobulin fractions in column buffer (0.15 M NaCl - 20 mM potassium phosphate (pH 7.2) - 1 mM NaN_3) were applied to the column and allowed to circulate at room temperature for 3 hrs at a rate of 1 column volume/hr. Bound antibodies were eluted with 1 M propionic acid - 0.15 M NaCl and immediately neutralized with an equal volume of 1.5 M K_2HPO_4. The bound fractions were dialyzed overnight at 4°C against PBS (0.1 M NaCl - 10 mM potassium phosphate (pH 7.2) - 1 mM NaN_3) and the antibodies were precipitated by ammonium sulfate (25 g/100 ml). After centrifugation at 4000 xg for 30 min., the antibodies were dissolved in a small amount of PBS and dialyzed versus PBS or column buffer.

All antibodies used for immunochemical procedures were affinity purified. Antibodies binding to the fast myosin column after application of crude anti-fast or anti-mixed-myofiber immunoglobulins were used in procedures utilizing anti-fast antibodies. The non-bound fraction (eluted in the column void volume) from applied crude anti-mixed immunoglobulins was next applied to the mixed-myosin column. Antibodies binding to this column were specific for slow fiber myosin, any anti-fast myosin antibodies having been retained previously on the fast-myosin affinity column.

GEDELISA

GEDELISA (Lutz, et al., 1978) was performed to test the specificity of the antibodies for the myofibrillar proteins. Each antibody was tested against the myofibrillar proteins from the slow-tonic chicken anterior latissimus dorsi (ALD) and the myofibrillar proteins from fast-twitch pectoralis.

Identical loads of myofibrils were applied to 2 or more cylindrical stacking SDS gels (Laemmli, 1970) and the proteins separated by electrophoresis. One gel was stained with Coomassie Blue and the location of the protein band recorded with a densitometer scan. The other gel was sliced into 1 mm segments using a mechanical gel slicer (Hoefer Scientific, San Francisco, CA). Each gel slice was incubated overnight in a well of a polystyrene tissue culture plate (Costar #3596, Bellco Glass) containing 150 µl of 0.1 M Na_2CO_3 pH 9.6. This resulted in the elution of protein from the gel slice and subsequent protein absorption to the well wall. The plate had previously been treated with glutaraldehyde to minimize protein desorption (Howell et al., 1981) during the enzyme-linked immunosorbent assay (ELISA) which followed.

An ELISA (Engvall and Perlman 1972) was performed on the plate; the separated myofibrillar proteins, now adsorbed to the well walls, being the antigens to be tested for reaction with antibody. Each well was incubated for 30 min. at room temperature (RT) with 200 µl of 3 mg/ml bovine serum albumin (BSA) in standard saline (SS; 0.9% NaCl -0.05 M potassium phosphate (pH 7.4) - 1 mM NaN_3) to block any remaining reactive sites. Three rinses of 5 min. each with .05% Tween 80 - 1% BSA - SS followed. Primary antibody (1° Ab; 100 µl of 1 µg/ml) was added to each well, incubated at RT for 2 hrs and then rinsed to remove any 1° Ab not specifically bound to the adsorbed protein.

Alkaline phosphatase conjugated goat anti-rabbit secondary antibody (2° Ab; 100 µl of 1:1500 dilution)(Miles Labs., Elkhart, IN) was pipetted into each well, incubated at RT for 2 hrs, and rinsed. The enzyme substrate, p-nitrophenyl phosphate (Sigma Chem. Co., St. Louis, MO) was next added to each well (100 µl of 10 mg/ml) and allowed to react for 3 hrs after which the reaction was stopped by the addition of 50 µl of 3 N NaOH. The presence of a colored product in a well, measured spectrophotometrically on a micro-ELISA reader (MR 580, Dynatech) indicated reactivity between 1° Ab and the myofibrillar protein in that well.

Indirect Immunofluorescence

Indirect immunofluorescence and histochemical staining for myofibrillar ATPase and SDH were performed on serial 8 µ thick sections of a mixed myofiber region of chicken pectoralis which had been frozen in liquid nitrogen cooled isopentane. The cryostat cut sections were picked up on albumin coated slides and allowed to air dry for 30 min.

For indirect immunofluorescence each section was incubated with a drop of 1° Ab (anti-fast, 0.04 mg/ml or anti-slow, 0.05 mg/ml) for 30 min. at RT, and then rinsed 3 times for 10 min. each in PBS. An incubation with 2° Ab (fluoroscein conjugated goat anti-rabbit Ig G) (Miles Labs) for 30 min. followed, and the sections were rinsed 4 times in PBS. The sections were mounted in 25% glycerol-PBS and coverslips sealed in place with fingernail polish. Sections were viewed under epifluorescence, and photographs were taken using Kodak Tri-X film with typical exposure times of approximately 30 seconds.

Histochemical Procedures

Myofibrillar ATPase was determined histochemically by the method of Suzuki (1976) with preincubations at pH 4.3 and 4.6 for 5 min. and at pH 9.4 and 10.3 for 20 min. Staining for SDH was according to the procedure of Dubowitz and Brooke, (1973).

Results

Histochemical Identification of Myofiber Types: Results from histochemical staining for ATPase and SDH are shown in Figures 1 and 2a and b. Type IRB myofibers have high ATPase activity after pH 4.3 and 4.6 preincubation and moderate ATPase activity after pH 9.4 and 10.3 preincubation. Type IRA myofibers have moderate ATPase activity after pH 4.3 preincubation and light ATPase activity after pH 4.6, 9.4, and 10.3 preincubation. Both IRA and IRB myofibers show moderate SDH activity with evenly distributed diformazan particles. Type II myofibers (IIR and IIW) show identical ATPase reactions: No ATPase activity after pH 4.3 preincubation, moderate activity after preincubation at pH 4.6, and strong ATPase activity after preincubations of pH 9.4 and 10.3. Type IIR myofibers have high SDH activity with subsarcolemmal aggregations of diformazan particles. Type IIW myofibers show a low to moderate reaction for SDH with some subsarcolemmal aggregation of particles.

Immunofluorescence

The myofiber typing by indirect immunofluorescence is pictured in Figures 2c and d. Only the slow-tonic myofibers (IRA and IRB) stain with anti-slow antibodies while only the fast-twitch myofibers (IIW and IIR) stain with anti-fast antibodies. Myofibers were never observed to react with both antibodies.

GEDELISA

GEDELISA results are illustrated in Figures 3a and b. Anti-fast antibodies react strongly with proteins from the pectoralis. The major reaction is with MHC, although two minor reactions with other high molecular weight proteins were observed. The reaction of anti-fast antibodies with ALD proteins shows a weak reaction only with MHC. Anti-slow antibodies react strongly with ALD proteins, showing a major reaction with MHC, and minor reactions with other high molecular weight proteins. The reaction of anti-slow antibodies with pectoralis proteins is very weak and only with MHC.

Discussion

Specificity of the anti-fast antibody for fast myofiber MHC and of anti-slow antibody for slow-myofiber MHC was established using GEDELISA. This is illustrated by large GEDELISA absorbance values, when using anti-fast or anti-slow antibodies, at an R_f value corresponding to the MHC from pectoralis (Figure 3a) and ALD (Figure 3b) respectively.

The fact that anti-fast antibodies show only a slight reaction with ALD MHC (Figure 3b) and that anti-slow antibodies show no reaction with pectoralis MHC (Figure 3a) is consistent with the conclusion of other researchers (Arndt and Pepe, 1975; Masaki, 1974) that fast and slow myofibers have antigenically distinct MHC's. The MHC's have also been shown to be structurally different on the basis of their electrophoretic migration in nondissociating gels (Hoh et al., 1976; Hoh, 1978). The minor GEDELISA reaction between anti-fast antibodies and the MHC from ALD myofibrils may be due to the presence of a few fast-twitch myofibers in this slow muscle (Arndt and Pepe, 1975; Gauthier and Lowey, 1977; Toutant et al., 1981).

The histochemical staining for SDH and for myofibrillar ATPase at several preincubation pH's (Figures 1 and 2a and b) were performed in order to obtain sufficient evidence to establish the identity of chicken skeletal muscle myofibers within already published criteria (Khan, 1976; Suzuki, 1982; Toutant et al., 1981). We have used the nomenclature of Khan, 1976 for classifying myofibers, but Table 1 allows comparison with other nomenclature systems (see also Toutant et al., 1981).

Table 2 provides a summation of our observations using the histochemical and immunofluorescent staining procedures. We concluded that staining for myofibrillar ATPase using a pH 4.3 preincubation together with staining for SDH would effectively establish histochemical myofiber type identity. Anti-fast antibodies only stained the histochemically fast-twitch myofibers (IIW and IIR) and anti-slow antibodies only stained the histochemically slow-tonic myofibers (IA and IB). The observation that an individual myofiber never stained with both antibodies indicated the homogeneity of each myofiber for a specific MHC isozyme.

The specificity of each antibody for its homologous MHC isozyme as illustrated by GEDELISA, combined with the homogeneity observed in immunofluorescence of each individual myofiber for a MHC isozyme, have proven the antibodies accurate and reliable in myofiber typing. The observed agreement between immunofluorescent myofiber typing and histochemical myofiber typing supports the use of ATPase as an accurate indicator of myofiber types, fast-twitch or slow-tonic, in the chicken.

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Table 1. Correspondence Between Nonenclature Systems Used To Type Various Chick Muscles

Fiber Type	Khan 1976 (Soleus)	Toutant et al., 1981 (Biventer Cervis)	Suzuki et al., 1982 (Thigh Muscles)
Slow-tonic Oxidative	I Red B[1]	β_2 Red[1]	SS or SM[2]
Slow-tonic Oxidative	I Red A	β_1 Red	I
Fast-twitch Oxidative Glycolytic	II Red	α Red	IIA
Fast-twitch Glycolytic	II White	α White	IIB

[1]In our hands I Red B appears to correspond to β_2 Red.

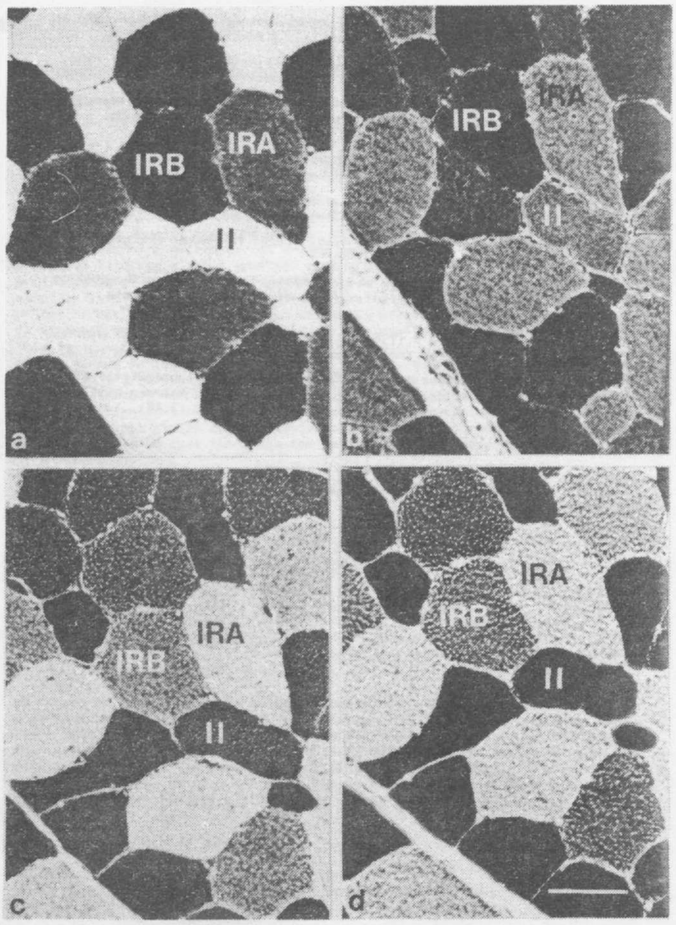
[2]SS appears to correspond to β_2 Red; SM appears to correspond to I Red B (Suzuki et al., 1982).

Table 2. Histologic and Immunologic Characteristics of Chick Pectoralis Fiber Types

Fiber Type	ATPase Pre-incubation pH 4.3	4.6	9.4	10.3	SDH	Rxn with Ab Anti-fast	Anti-slow
IRB	+++[a)]	+++	++	++	Moderate[b)] and Disperse	--	+++
IRA	++	+	+	+	Moderate and Disperse	--	+++
IIR	--	++	+++	+++	High and Subsarcolemmal	+++	--
IIW	--	++	+++	+++	Low and Subsarcolemmal	+++	--

[a)]Relative extent of reaction at a preincubation pH: +++ strong; ++ moderate; + weak; -- absent.

[b)]Extent of reaction and localization of diformazan particles.



Legends for Figures

Fig. 1. Serial cross-sections of the mixed-myofiber region of chicken pectoralis stained for histochemical demonstration of ATPase at preincubation of: (a) pH 4.3; (b) pH 4.6; (c) pH 9.4 (d) pH 10.3. Examples of myofiber types are labeled. Bar equals 50 μm.

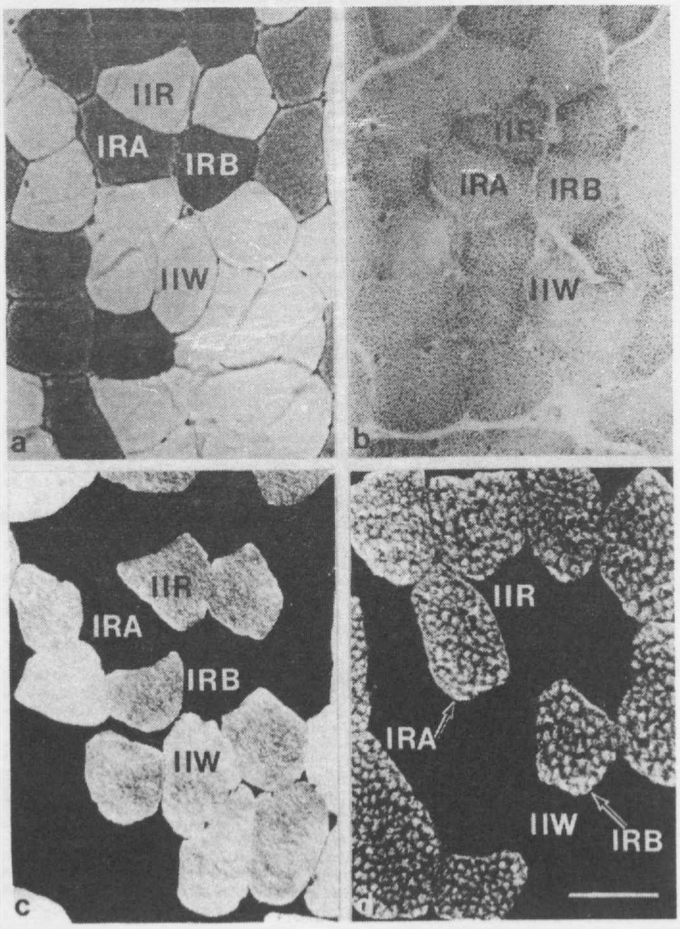


Fig. 2. Serial cross-sections of the mixed-myofiber region of chicken pectoralis. Histochemical demonstration of: (a) ATPase, preincubation at pH 4.3; (b) SDH. Immunofluorescence using (c) anti-fast antibodies; (d) anti-slow antibodies. Examples of myofiber types are labeled. Bar equals 50 μm.

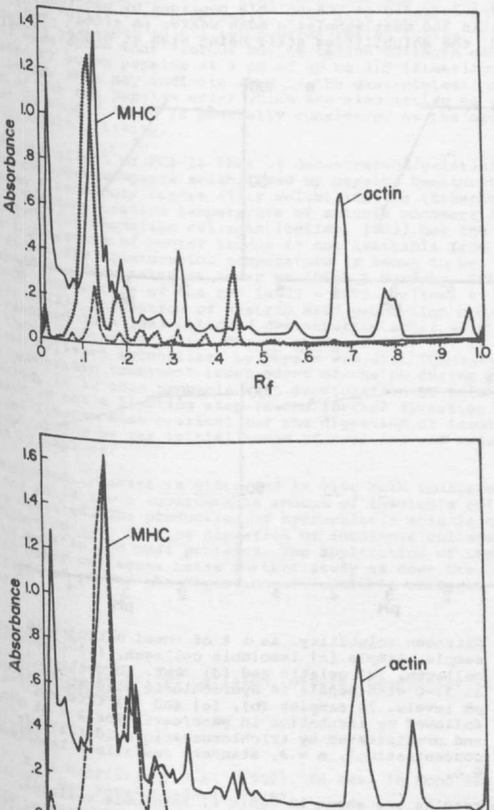


Fig. 3. GEDELISA results on (a) ALD myofibers; (b) pectoralis myofibrils. Solid lines are densitometer scans at 560 nm. Dashed lines are ELISA results using anti-fast antibodies. Dotted lines are ELISA results using anti-slow antibodies. R_f values are calculated as the distance from the origin of the separating gel to a specific point divided by the distance from the origin of the separating gel to the dye front.