ACTA FAC MED NAISS

UDC 599.323.4:591.473

Original article

ACTA FAC MED NAISS 2008; 25 (1): 41-45

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IDENTIFICATION OF FIBER TYPES IN RAT SKELETAL MUSCLE BASED ON THE USE OF CATIONIC DYES IN MYOFIBRILLAR ADENOSINE TRIPHOSPHATASE REACTION

SUMMARY

The histochemical reaction for myofibrillar adenosine triphosphatase (mATPase) is widely used method in typing skeletal muscle fibers. The mATPase reaction allows a qualitative and quantitative evaluation of fiber types in normal, diseased, or experimentally altered muscles.

The aim of this study was to investigate the reproducibility and validity of cationic dyes' use in the mATPase reaction for identifying fiber types in the rat soleus muscle by the method proposed by Doriguzzi et al.

The soleus muscle was removed from 10 adult Wistar albino rats of both sexes under ether anesthesia. Serial frozen cross-sections 10 µm thick were prepared and reacted for mATPase after preincubation at pH 9.4, 4.5 and 4.3, and for modified mATPase with the use of Toluidine blue (TB) and Methylene blue (MB) in post-incubation treatment of sections.

The mATPase reaction demonstrated by TB and MB displayed a clear cut differentiation of muscle fiber types corresponding to type I, IIA and IIC fibers, obtained by mATPase reaction with ammonium sulfide. The method based on the use of cationic dyes in mATPase reaction appears to be fast, reproducible and valid.

However, in our experience, the intensity of coloration of muscle fibers decreased by time so that the distinction between fiber types become difficult, which stands for the main disadvantage when compared to the conventional mATPase method.

Key words: **fiber types, rat skeletal muscle, mATPase histochemistry, ammonium sulfide, cationic dyes**

INTRODUCTION

The histochemical reaction for myofibrillar adenosine triphosphatase (mATPase), originally proposed by Brooke and Kaiser (1), is perhaps the most widely used method for identifying the types and subtypes of fibers in skeletal muscle. The reaction is based on splitting of the terminal phosphate from ATP, which combines with calcium present in the incubation medium to form an insoluble salt. Thereafter, cobalt is exchanged for calcium and cobalt phosphate is demonstrated by ammonium sulfide. The mATPase reaction after alkaline preincubation differentiates muscle fibers into two types: I (slow-twitch) and II (fast-twitch). With preincubation at various acid pH, different types of fast-twitch muscle fibers can be identified, based on sensitivity of their mATPase to acid (1, 2). Several other treatments have been used for identifying the major fiber types (3-7). The method of Doriguzzi at al. (5) based on the use of metachromatic dyes, namely Azure A and Toluidine blue, to demonstrate the precipitated phosphate arose interest because they suggested it was speedy, easy,

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and valid for typing the human muscle fibers.

The activity of mATPase is known to be affected by many factors including the time, temperature, pH, type of buffer, and ionic composition of the preincubation medium (1-4, 6,7). Therefore, unless the conditions for the histochemical demonstration of the mATPase are rigidly controlled, comparisons between results from different laboratories may be difficult or even meaningless.

The aim of this study was to investigate whether the use of cationic dyes in mATPase reaction could provide a basis for differentiation of the rat soleus muscle fibers in comparison to conventional mATPase method.

MATERIAL AND METHODS

The soleus muscle was removed from 10 adult Wistar albino rats of both sexes under ether anesthesia. The muscle samples were rapidly frozen in isopentane cooled by liquid nitrogen. Serial cross-sections 10 μm thick were cut in a cryostat at -20°C and air-dried.

The incubation medium was prepared according to Dubowitz and Brooke (2) - 25 mg ATP-disodium salt dissolved in 2 ml sodium barbital 0.1 M, 1 ml calcium chloride 0.18 M, 7 ml distilled water, and adjusted at pH 9.4 with NaOH 0.1 N. Alkaline preincubation was carried out at pH 9.4 in sodium barbital buffer. Sodium acetate-acetic acid buffers adjusted at pH 4.5 and 4.3 were utilized for preincubation to obtain the reversal mATPase reaction. Serial cross-sections were processed according to the following procedures:

I. Myofibrillar ATPase reaction demonstrated by using ammonium sulfide.

The reaction was carried out according to the method described by Dubowitz and Brooke (2). The preincubation at pH 9.4 lasted for 15 min, and at pH 4.5 and 4.3 for 5 min at room temperature.

II. Myofibrillar ATPse reaction demonstrated by using cationic dyes.

1. Some sections were preincubated at pH 9.4 (15 min) and others at pH 4.5 and 4.3 (5 min) at room temperature in the same buffers as reported above.

2. The incubation time ranged from 1-45 min in the medium at 37°C.

3. Sections were stained in freshly prepared Toluidine blue (TB) 1% in distilled water (or in freshly prepared 1% water solution of Methylene blue, MB) for 10 sec to 2 min.

4. Sections were rinsed with distilled water, observed under the microscope, then rapidly dehydrated and mounted in Canada balsam.

Control sections were treated as reported above (II), but the incubation medium was prepared without ATP as substrate.

RESULTS

Results of different staining procedures in the identification of muscle fiber types are summarized in *Table 1*. The slow soleus muscle of the rat contains only two (type I and IIA) of the three major types (I, IIA and IIB) of muscle fibers. The standard mATPase reaction with preincubation at pH 9.4 differentiates darkly stained type II fibers and lightly stained type I fibers *(Figure 1a)*. This reaction is reversed following preincubation at pH 4.3 (see later). In preincubation at pH 4.5, the activity of acid-sensitive mATPase in type IIA fibers was inhibited. However, among type II fibers, there was a certain number of fibers with intermediate activity. These fibers were designated as type IIC, in which the reaction is not inhibited by preincubation at either pH 4.5 or 4.3, having an intermediate staining between type I and IIA fibers *(Figure 1b)*. On the other hand, type I fibers showed enhanced acid-stable mATPase activity after both pH 4.5 and 4.3 preincubations *(Figure 1b)*.

Sections stained with TB and MB displayed a clear cut differentiation of muscle fibers after both alkaline and acid preincubations *(Figure 1c and 1d)*. It should be pointed out that the fibers with high content of phosphate (i.e. type II at pH 9.4) were orthochromatic, while those with low content of phosphate were metachromatic in non-dehydrated sections stained by TB (not shown). This effect with MB was not clearly seen. Alcohol dehydration caused the loss of metachromasia.

Table 1. Staining pattern of fiber types in the rat soleus muscle determined by mATPase histochemistry by using ammonium sulfide and cationic dyes

Preincubation		Time	Muscle fiber type		
	pH	(min)	I	IIA	IIC
	9.4	15			
Ammonium sulfide			light	dark	dark
Toluidine blue			light blue	blue	blue
Methylene blue			light green-blue	green-blue	green-blue
	4.3*	5			
Ammonium sulfide			dark	light	intermediate
Toluidine blue			blue	light blue	intermediate
Methylene blue			green-blue	yellow	intermediate

**Similar staining pattern was obtained after preincubation at pH 4.5 for 5 min, although the distinction between fiber types was better after pH 4.3 preincubation.*

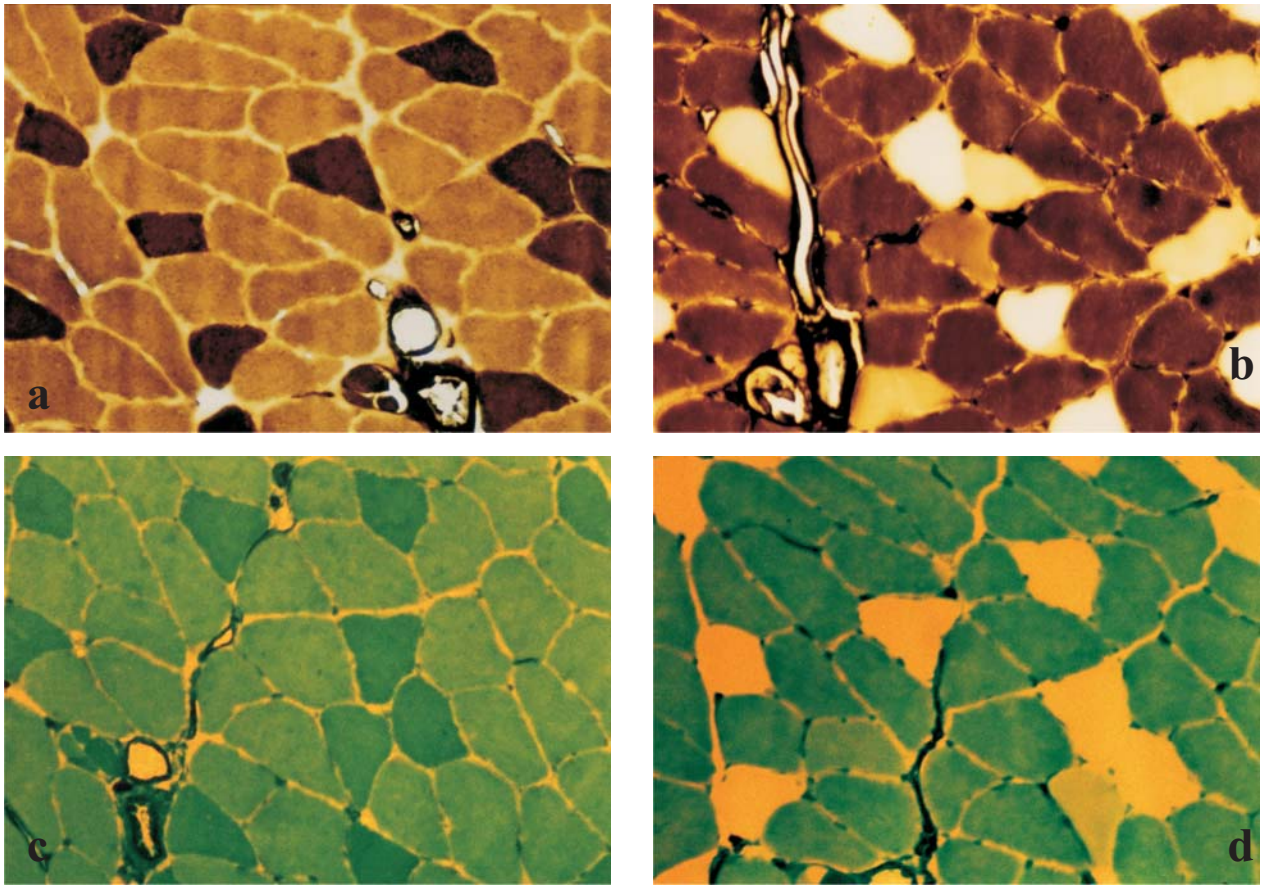

Figure 1. Serial cross-sections of the rat soleus muscle stained for mATPase after preincubation at pH 9.4: (a) Ammonium sulfide, (c) Methylene blue.
Two fiber types are identifiable - darkly reacting type II fibers and lightly reacting type I fibers.
Sections stained for mATPase after peincubation at pH 4.3: (b) Ammonium sulfide, (d) Methylene blue.
Note the reversal reaction of both type IIA and type I fibers and appearance of type IIC fibers with intermediate reaction. Bar =50 μm.

In sections, after rinsing in water and dehydration, both TB and MB differentiated muscle fiber types with intensity of coloration that is proportional to the content of released phosphate. In all sections stained by TB or MB, excess rinsing and excess dehydration caused progressive loss of fiber type distinction.

The time periods of incubation in the reaction medium and in the dye were tested. The combinations producing consistent contrast among types of muscle fibers, comparable to the conventional mATPase reaction, were determined. The best results for standard mATPase were obtained after 5 min in the incubation medium and 15 sec in the dye, whereas for mATPase with acid, preincubation (pH 4.5 and 4.3) after 15 min in medium and 15 sec in the dye. The comparison of serial cross-sections stained with cationic dyes and with ammonium sulfide revealed a concordant staining reaction corresponding to type I, IIA and IIC fibers *(Figure 1a-b and 1c-d)*. Control sections incubated without substrate showed no distinction of fiber types.

DISCUSSION

The mATPase reaction has been useful and extensively used in the identification of skeletal muscle fiber types. This reaction allows a qualitative and quantitative evaluation of fiber types in normal, diseased, or experimentally altered muscles (2, 3, 8-13). It is known that histochemical demonstration of mATPase is markedly influenced by pH of the preincubation medium, among many other factors (1-4, 6, 7). Generally, typing of muscle fibers based on the sensitivity of mATPase to acid and alkaline activation and inactivation is related to the presence of myosin heavy chain isoforms that can be identified immunohistochemically, electrophoretically, and by in situ hybridization (14-16). Thus, it was shown that skeletal muscles of adult rats express four myosin heavy chain isoforms, one slow and three fast, whose distribution correlates with fiber types delineated using mATPase histochemistry (14).

The staining reaction of fiber types on mATPase after preincubation at various pH values

depends upon the amounts of phosphate liberated from ATP. It is known that acidic (anionic) groups, such as phosphate, can be revealed by cationic dyes (17). Substances that carry acidic groups can react with certain cationic (metachromatic) dyes, such as TB, to produce a color which is different to that normally exhibited by the dye (i.e. purple) (18). The phenomenon of metachromasia depends on several factors including the polymerization of dye, the relationship between acidic groups and dye concentration, the pH, the type of dye and the technique employed (17-19).

In this study, the use of cationic dyes in post-incubation treatment of sections to stain phosphate liberated from ATP by mATPase was shown to allow the distinction between fiber types, as previously proposed by Doriguzzi et al. (5). Namely, both TB and MB differentiated muscle fiber types, with intensity of coloration that is proportional to the content of phosphate, corresponding to those obtained by ammonium sulfide, despite the fact that MB gave no clear effect of metachromasia.

The use of TB and MB was proved to be valid for distinction between fiber types not only in the slow soleus muscle, but also in fast muscles of the rat (unpublished results). We also investigated whether the use of TB and MB in mATPse and alkaline phosphatase (calcium-cobalt sulfide) reactions is valid in evaluation of experimentally altered rat skeletal muscle fibers. Preliminary results are satisfactory.

Although a close correlation between the fiber types classified by the conventional mATPase and by mATPase with use of cationic dyes was found in human muscle (5) and in the rat soleus muscle, appropriate timing of incubation in the reaction medium and in the dye were not identical. For human muscle, these time periods were 2-4 min and 10 sec, respectively (standard mATPase) and 10-15 min and 10 sec (reversal mATPase) (5), whereas for the rat soleus muscle, the best time periods were 5 min and 15 sec (standard mATPase) and 15 min and 15 sec, respectively (mATPase with preincubation at pH 4.5 and 4.3).

We observed that the incubation for 5 and 15 min allows better control intensity of muscle fibers coloration in dye under the microscope.

CONCLUSION

In conclusion, the method based on the use of cationic dyes in mATPase reaction is fast and easy, and can be used as an alternative to the conventional multiple steps mATPase method.

However, in our experience, the intensity of coloration of muscle fibers decreased by time so that the distinction between fiber types became difficult, which stands for the main disadvantage when compared to the conventional mATPase method.

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IDENTIFIKACIJA TIPOVA VLAKANA U SKELETNOM MIŠIĆU PACOVA PRIMENOM KATJONSKIH BOJA U REAKCIJI MIOFIBRILARNE ADENOZIN TRIFOSFATAZE

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SAŽETAK

Histohemijska reakcija za dokazivanje miofibrilarne adenozin trifosfataze (mATPaze) je metoda sa širokom primenom za tipizaciju skeletnih mišićnih vlakana. Reakcija mATPaze omogućava kvalitativnu i kvantitativnu evaluaciju tipova vlakana u normalnim, obolelim ili eksperimentalno oštećenim mišićima.

Cilj ovog rada bio je da se ispita reproducibilnost i validnost primene katjonskih boja u reakciji mATPaze za identifikaciju tipova vlakana u musculus soleusu pacova metodom koju su predložili Doriguzzi i saradnici.

Musculus soleus je uklonjen pod etarskom anestezijom od 10 Wistar albino pacova oba pola. Na serijskim poprečnim presecima, debljine 10 µm, trenutno smrznutog materijala primenjena je histohemijska metoda na mATPazu sa preinkubacijom pri pH 9,4; 4,5; i 4,3 i modifikacija mATPaze uz korišćenje toluidin plavog (TP) i metilen plavog (MP) u postinkubacionom tretmanu. Reakcija na mATPazu vizualizovana TP i MP pokazala je jasnu diferencijaciju tipova mišićnih vlakana, koji su odgovarali vlaknima tipa I, IIA i IIC identifikovanim reakcijom na mATPazu sa amonijum sulfidom. Metoda koja je bazirana na korišćenju katjonskih boja u reakciji na mATPazu je brza, reproducibilna i validna.

Međutim, prema našem iskustvu, intenzitet bojenja vlakana vremenom opada tako da je razlikovanje tipova vlakana otežano, što čini glavni nedostatak ove u odnosu na konvencionalnu mATPaza metodu.

Ključne reči: **tipovi vlakana, skeletni mišić pacova, mATPaza, amonijum sulfid, katjonske boje**