Separation by Gel Electrophoresis under Non Dissociating Conditions (PPi PAGE) of the Native Isoforms of Myosin - A Technical Note

Anne d'Albis and Chantal Janmot

Laboratoire de Biologie Physicochimique, URA CNRS 1131, Université Paris-Sud, Orsay, France.

Abstract

Polyacrylamide gel electrophoresis of myosin under non dissociating conditions, in the presence of sodium pyrophosphate (PPi PAGE), is a convenient technique for analyzing a muscle myosin isoform content. The experimental conditions for this analysis are reported. Examples of myosin isoform separation, quantitative analysis by densitometry, and ATPase activity determination on gel are described.

Key words: native myosin isoform, gel electrophoresis.

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Myosin is one of the major proteins of muscle and motile cells; together with actin, it catalyses the hydrolysis of ATP, which generates the energy necessary for movement.

Myosin is a large protein, composed of 2 heavy chains and 4 light chains; the C terminal portions of the 2 heavy chains are coiled to form a fibrous tail, and their N terminal portions, in association with the light chains, form 2 globular heads. The tail forms the structural part of the myosin molecule and the heads are responsible for its enzymatic activity.

There are a number of isoforms of myosin, which differ either by their heavy chains, their light chains, or both; the differences between the sequences of the chains do not exceed generally 20%, and may be only 2 to 3%. These differences normally allow the separation of the myosin isoforms by electrophoretic procedures. Several denaturing procedures (in the presence of SDS, urea, or phenol-acetic acid), are available to separate the various myosin heavy and light chains. We describe here a procedure, which allows the separation of different myosin isoforms in their native state.

Electrophoresis, and in particular polyacrylamide gel electrophoresis, is normally performed at low ionic strength, to avoid overheating. However, at physiological ionic strength, myosin molecules assemble to form thick insoluble filaments, which aggregate at the top of gels and prevent the separation of myosin isoforms.

To obtain migration of myosin in its native form, it is necessary to use sodium pyrophosphate (which is a dissociating buffer for myosin filaments) as an electrophoresis buffer, to circulate this buffer between the two electrode compartments, and to perform the electrophoresis run near 0°C [1, 4, 5, 3].

Experimental

Myosin samples

It is not necessary purify the myosin for separation of the myosin isoforms present in muscle samples. Small pieces of muscle should simply be washed with a low ionic strength buffer (A), which will remove all the soluble cytoplasmic proteins; myosin (and other myofibrillar proteins) is then extracted with a high ionic strength buffer (B).

All the steps of the preparation are performed at 4°C, either on ice or in a cold room.

- A freshly removed muscle or a muscle which has been conserved in liquid nitrogen is incubated (chilled or thawed) on ice. Fat and tendons are removed and the muscle (down to a few mg) is wiped and then weighed in centrifuge tubes.
- Five volumes of cold wash buffer A are added to the muscle, which is cut into small pieces with thin scissors (large muscles should be distributed into several centrifuge tubes).
- Centrifuge for 10 mn with a bench centrifuge and discard the supernatant.
- Add to the pellet 3 volumes of cold extraction buffer B and shake gently for 30 mn; centrifuge for 15 mn.

- Measure the volume of the supernatant and add an equal volume of glycerol. This myosin extract can be stored at - 20°C.
- Before electrophoresis, dilute the myosin extract (between 5 and 100 times, depending on muscle, age of the animal, its pathological state...) with sample buffer C.

Wash buffer A:
20 mM NaCl
3.4 mM PO_4H_2Na
1.6 mM PO_4HNa_2
1 mM EGTA
→ pH 6.4 (without adjustment)

Extraction buffer B:
100 mM $Na_4P_2O_7$ pH 8.5 (pH adjusted with HCl N)
5 mM EGTA
1 mM DTT

Sample buffer C:
20 mM $Na_4P_2O_7$ pH 8.5
30% Glycerol
0.1% Mercaptoethanol
2 mM $MgCl_2$
a few grains of Bromophenol Blue

Electrophoresis equipment

Electrophoresis is performed at a temperature close to 0°C for 16 to 24 hours (in general 22 hours). The electrophoresis apparatus must therefore be cooled by an external fluid and the electrophoresis buffer has to circulate between the two electrode chambers. The electrophoresis is performed at a relatively high ionic strength ($\mu = 0.35$), and this generates heat; the temperature of the cooling fluid (an alcool-water mixture) must be set at - 15°C to - 20°C (depending on the ambient temperature), to maintain a temperature close to 0°C during the whole run.

Required apparatus:
- A cryostat, capable of reaching - 25°C, connected to the electrophoresis apparatus
- An electrophoresis apparatus with a cooling device and circulation between the two electrode chambers.
- A power pack (90 V).
- A scanning densitometer, if quantitative results or ATPase measurements are required.

Electrophoresis
- Cool the electrophoresis buffer (D) to 0°C- 2°C in the electrophoresis apparatus by connecting the apparatus to the cryostat.
- Gels. Either tube or plate gels can be used. Resolution is better with tube gels, but plate gels are more convenient in case of subsequent immunoblotting. Prepare the gels with solution E at room temperature. The gels set after about 5 to 10 mn and are ready for use after one hour.
- Perform a preelectrophoresis for 15 mn at a constant voltage of 90V for tube gels (6 cm height x 0.5 cm diameter) and at a constant voltage of 60V for plate gels (17 cm width x 7 cm height x 0.07 cm thickness).
- Load the myosin samples (0.2 to 1 µg per myosin band) and perform the electrophoresis at the same voltages, for 22 hours with tubes and 16 hours with plates.
- Extrude the gels and stain (F), destain (G and H), and keep (I) as usual.

Electrophoresis buffer D:
20 mM $Na_4P_2O_7$
10% Glycerol
0.01% Mercaptoethanol
2 mM $MgCl_2$
adjust the pH to 8.8 (room temperature) with concentrated HCl

Gel buffer E:
20 mM $Na_4P_2O_7$ (from 100 mM $Na_4P_2O_7$ pH 8.5 stock solution)
3.88% Acrylamide + 0.12% bis-acrylamide
10% Glycerol
0.05% Ammonium persulfate
0.2% TEMED

Staining solution F:
0.2% Coomassie Brilliant Blue
50% Ethanol
10% Acetic Acid

Destaining solutions G and H: alternatively
30% Ethanol
5% Acetic Acid
and 5% Ethanol
5% Acetic Acid

Conservation solution I:
7.5% Acetic acid

Myosin ATPase activity on gels (according to [4])

After 20 hours of electrophoresis (in general next morning), stop the circulation of electrophoresis buffer between the two electrode chambers and replace the upper compartment buffer with buffer J. Resume electrophoresis for 2 hours.

Incubate the extruded tube gels with shaking in ATPase assay buffer K at 37°C and follow the appearance of white bands of calcium phosphate precipitate, which correspond quantitatively to the hydrolysis of ATP.

Electrophoresis ATPase buffer J:
190 mM Glycine
20 mM Tris
5 mM ATP
10% Glycerol
adjusted to pH 8.8 with NaOH N

ATPase assay buffer K:
190 mM Glycine
600 mM KCl
15 mM $CaCl_2$
25 mM Tris
0.05% Mercaptoethanol
and 5 mM ATP immediately before use.

Examples (reproduced from [2])

As an example of myosin isoform separation by non dissociating gel electrophoresis (PPi PAGE), fig. 1 shows the separation of the various myosin isoforms present in the masseter muscle of rabbits at various ages. At 12 days postnatal, there is a mixture of neonatal and adult fast

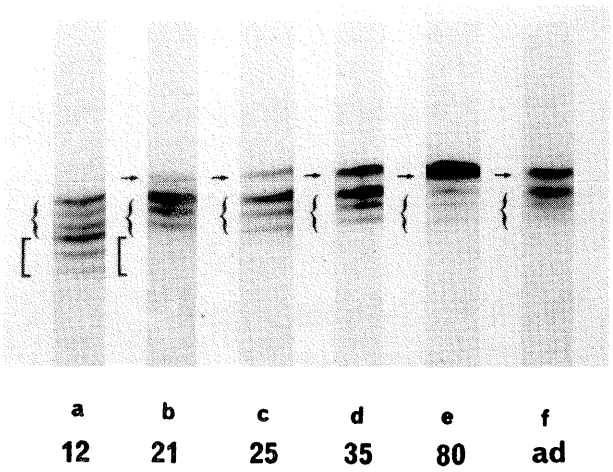

Figure 1. Electrophoresis under non-dissociating conditions of the myosin isoforms present in the masseter during rabbit development.
a: 12 days; b: 21 days; c: 25 days; d: 35 days; e: 80 days; f: adult.
The symbols designate the different isoforms.
→ ventricular-type isoform V 1
{ type II isoforms
[neonatal isoforms.
(reproduced from Fig 7 in [2])

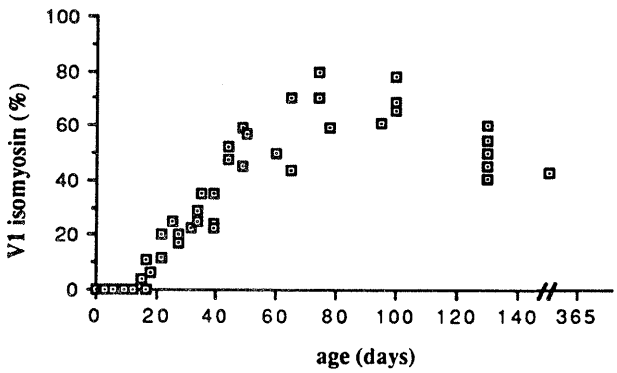

Figure 2. Variation with the age of rabbit of the proportion of the ventricular-type V1 myosin isoform present in the masseter (reproduced from Fig. 8 in [2]).

myosin isoforms; at 21 days, the neonatal isoforms have almost disappeared, and a cardiac type V1 myosin isoform is present; at 80 days, the cardiac type isoform predominates, but adult fast myosins are still visible.

The respective isoforms can be quantified by densitometry, as shown in figure 2, where the variation of the proportion of the cardiac type V 1 myosin isoform with age is displayed.

Relative Ca^{2+}-ATPase activities of myosin isoforms can be assessed from non-dissociating gels as shown in figure 3. Comparison between the protein (a) and the ATPase (b) scans shows that in the conditions of the experiment, the adult slow type myosin displays no activity, while the V1 cardiac type isoform displays a higher ATPase activity than the adult fast myosin isoforms.

Gel electrophoresis of myosin under non dissociating conditions presents several advantages. It is easy to perform, it does not require purification of myosin, and it necessitates only small quantities of muscle. It allows a quantitative analysis of the different myosin isoforms present in a muscle, as well as their relative Ca^{2+}-ATPase activities. Subsequent immunological analysis with specific antibodies is possible. Non dissociating electrophoresis is primarily analytic, but can also be used as a preparative method for diverse applications; after electrophoresis, each myosin band can be excised and its light and heavy chain content determined on a second denaturing gel. Myosin from several bands can also be pooled for antibody preparation. Gel electrophoresis of myosin under non dissociating conditions is thus a valuable technique for all those working with myosin.

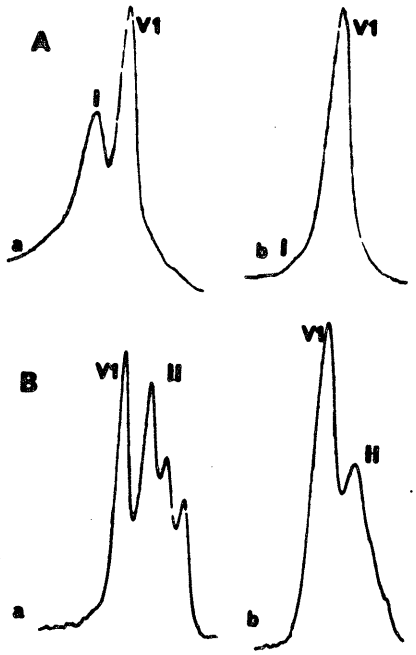

Figure Figure 3. Separation by electrophoresis under non-dissociating conditions of rabbit myosin isoforms and determination of Ca^{2+}-ATPase activity. Densitometric scans.
A: coelectrophoresis of soleus type I myosin isoform and of masseter (anterior deep and middle masseter) ventricular-type V 1 myosin isoform.
B: electrophoresis of masseter ventricular-type V1 myosin isoform and type II myosin isoforms. a: protein staining; b: Ca^{2+}-ATPase activity, at pH 8.8 (adapted from Fig. 3 in [2]).

Address correspondence to:

Anne d'Albis, Laboratoire de Biologie Physico-chimique, Université Paris-Sud, Batiment 433, 91405 - Orsay, France, tel. 33 169 4164 28, fax 33169 85 3715.

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