Straightforward Identification of MHCemb by Hydroxylamine-Peptide Mapping

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Abstract

MHCemb is a useful marker of skeletal muscle regeneration in adult muscle, and therefore it is used to reveal/quantify incoming muscle damage. Immunochemical analyses are routinely performed in muscle biopsies, though cross-reactivity of polyclonal or monoclonal antibodies with fast-type adult MHC may undermine results. We here describe a peptide mapping approach to MHCemb identification based on SDS PAGE of hydroxylamine-digest of muscle contractile proteins. The method produces high molecular weight peptides, which are peculiar of each MHC isoform, though at some expenses of sensitivity. The method is substantiated by immunoblotting and shown to be effective with human MHCemb, which could be identified among the contractile proteins solubilized with SDS from a few muscle cryostat sections.

Key words: MHCemb, embryonic myosin, rat, human isomyosins, muscle, regeneration, damage, healing, immunochemistry, peptide mapping, hydroxylamine.

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In sarcomeric muscles of high vertebrates myosin is an esapolypeptide made of two heavy and four ligth chains. Gel electrophoresis in nondenaturing conditions separates the slower running band of slow isomyosin from the faster running bands of fast isoforms, while embryonic and neonatal isomyosins run with or ahead of the fast bands [13]. Two dimensional polyacrylamide gel electrophoresis (2D PAGE) shows that both fast and slow isomyosins contain three types of light chains (LC). The fast isomyosins contain two LC2F and different proportions of the alkali light chains LC1F and LC3F. The slow isomyosin contains two LC2S and different proportions of the alkali light chains LC1S and LC1'S [8]. The embryonic isomyosin of skeletal muscle contains, in addition to two LC2F, a pair of a peculiar alkali LCemb [9]. LCemb and LC2F genes continue to be expressed in cardiomyocites of the adult atrium, whereas ventricular myosin contains LC1S and LC2S, but not LC1'S [10, 14]. LCemb is therefore a usefull marker of embryonic myosin in skeletal muscle, but its low proportion in the native myosin (5% by weight), call in adult muscle specimens for more sensitive methods, i.e. analyses of myosin heavy chain (MHC) isoforms [11]. In mammals several sarcomeric myosin heavy chains have been identified: MHCEmb, MHCNeo, MHC2A, MHC2B, MHC2X, MHC1 or β-cardiac MHC, and α-cardiac MHC. The adult isoforms (MHC1, MHC2A, MHC2X and MHC2B) are usually segregated in distinct types of adult skeletal fibres; that is, each of them is present alone in single myofiber [5]. MHCemb and MHCneo are expressed in developing fibres during both ontogenesis and regeneration [22]. SDS PAGE of MHC is a powerful tool to identify MHC: slight but critical changes of the electrophoretic conditions allow the separation of the MHC isoforms of adult muscles into four distinct bands that run from the top of the gel in the following order: MHC2A, MHC2X, MHC2B and MHC1. The MHCemb and MHCneo run in between MHC2X and MHC2B, but resolution is quite poor in complex mixtures of MHC [5, 6, 17]. Immunochemical approaches simplify identification of MHCemb and are routinely used to demonstrate muscle damage/regeneration by immunocytochemistry and/or immunoblotting after gel electrophoresis of MHC [15, 20]. However a disturbing factor is a minor cross-reactivity of the anti-MHC monoclonals [4], which call for independent methods of MHCemb identification. We will show that hydroxylamine digestion of embryonic myosin and SDS PAGE analysis of its high molecular weight fragments

allow clear-cut identification of MHCemb in both rat and human muscle specimens.

Materials and Methods

We previously described in details the methods of myosin purification [11, 17] , SDS PAGE of MHC [12, 18, 19], and of MHC immunoblotting [2, 3]. A collection of the methods in bench-top-manual style is reported in [7]. Actual modifications are indicated in the figure captions. Specificity of antibodies: An anti-MHCemb mAbs (BF-G6) and an anti-MHC mAbs (IV8-F9) were used in this study. Antibodies were generous gifts of S. Schiaffino. Hydroxylamine digestion of MHC were performed essentially according to [1], with the following modifications. Preliminar experiments: A solution of 5,4 M guanidine and 1,8 M hydroxylamine (hydroxylamine-reagent) is prepared as for follows. Guanidine-HCl 2.5 g and 0.62 g of hydroxylamine are dissolved in 1.5 ml of HPLC-grade-water, then LiOH is slowly added till the pH rise to 10. The volume is adjusted to 5 ml with HPLC-grade-water. A

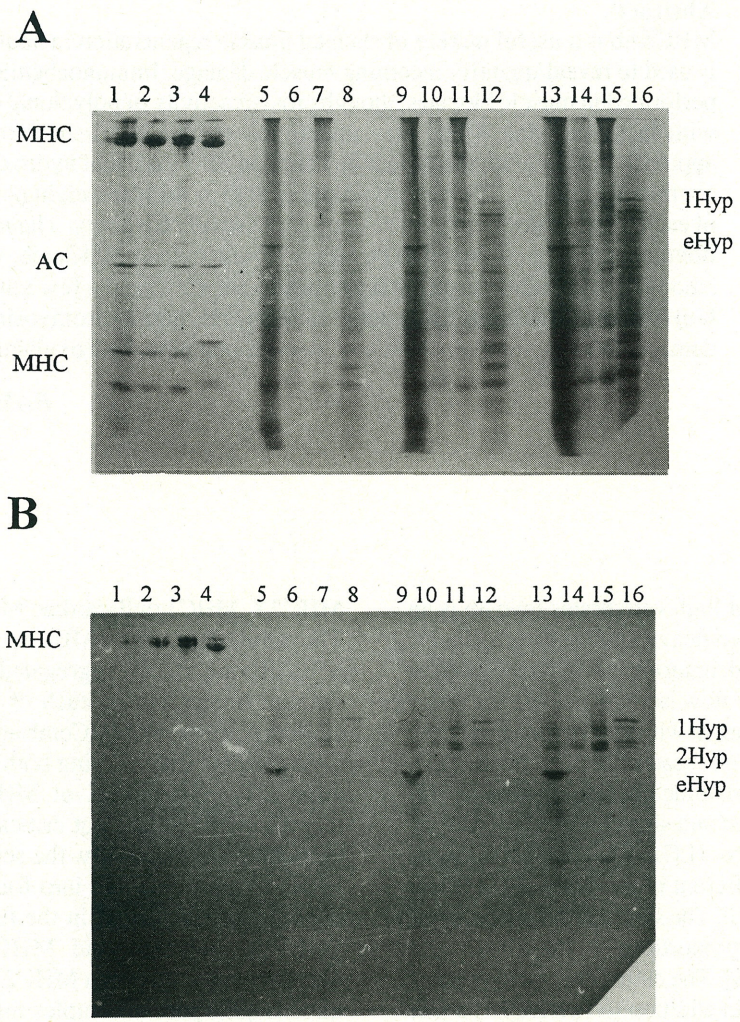


Figure 1. SDS PAGE and immunoblot analyses of MHC and of their hydroxylamine fragments from embryonic, neonatal, EDL and soleus rat muscles. A, Coomassie blue stain of SDS 10% PAGE analysis of MHC from: 10 µg of rat myosin purified from bulk skeletal muscle of 18-day embryos (1), 7-day neonatal leg muscles (2), adult EDL (3), adult soleus (4); hydroxylamine-peptides of embryonic myosin 20 µg (5), 30 µg (9) and 40 µg (13); hydroxylamine-peptides of neonatal myosin 20 µg (6), 30 µg (10) and 40 µg (14); hydroxylamine-peptides of EDL myosin 20 µg (7), 30 µg (11) and 40 µg (15); hydroxylamine-peptides of soleus myosin 20 µg (8), 30 µg (12) and 40 µg (16); B, Immunoblots of a slab identical to A reacted with Abs IV8-F9 a monoclonal anti-MHC (all the isoforms). MHC, myosin heavy chain; ac, actin; MLC, myosin light chains; eHyP, hydroxylamine-peptide of MHCemb; 1HyP, hydroxylamine-peptide of MHC1.

solution of muscle contractile proteins in 0.25 M NaCl, 50% (v:v) glycerol is mixed with the hydroxylamine-reagent in ratio of 1:2 by volume (one volume of contractile proteins with two volumes of hydroxylamine-reagent) or 1:1 by weight (one mg of contractile proteins with one ml of hydroxylamine-reagent). At 40°C a reaction time of two hours has been found optimal. The reaction was stopped with TCA according to [16].

Standard procedure: Hydroxylamine-digestion is performed as described above, but the contractile proteins are dissolved in 10% w:v (1.08 M) glycerol, 2.3% SDS, 5% v:v 2-mercaptoethanol, 62.5 mM Tris-HCl, pH 6.8 (Laemmli Sample Buffer, LSB), and denaturated by 3 min incubation in boiling water. SDS-solubilized-proteins are transferred to a dialysis tube and the hydroxylamine reagent is added. The reaction mixture is usually in the range of 0.1 -0.5 ml. After two hours at 40°C the hydroxylamine-digestion is terminated by two hour dialysis against bidistilled water at 40°C. The samples are mixed with one volume of LSB (final volume 0.2-1 ml).

We strongly recommend the use of dialysis-stopping to limit problems related to the handling of minute amounts of samples, usually in the range of 50-200 µl.

Results and Discussion

Hydroxylamine at alkaline pH cut glycine-asparagine bond and is therefore a relatively specific cleavage agent [1]. Digestion of EDL, soleus, neonatal and embryonic myosin with hydroxylamine produces a few high molecular weight peptides, whose SDS 10% PAGE patterns are remarkably different. The pattern of MHCemb digestion is characterized by a major peptide of about 55 kDa (indicated as eHyP) in Fig. 1A, lanes 5, 9, 13 , while digestion of the EDL myosin (Fig. 1A, lanes 7, 11, 15) produces a more heterogeneous pattern, which reflects the presence of at least four different isoforms of adult MHC [6, 17]. The electrophoretically separated MHC and their hydroxylamine digests were further characterized by immunoblots analyses. After transfer to nitrocellulose sheets, the MHC isoforms and their fragments were reacted with monoclonal antibodies reacting with all the MHC (mAbs IV8-F9). By this procedure only some of the peptides derived from MHC are revealed, while the large majority of them are negative (Fig. 1B, lanes 4-16).

Fig. 2 shows the results of a similar analysis but performed using an anti-MHCemb monoclonal. After transfer to nitrocellulose sheets, the MHC isoforms and their fragments were reacted with monoclonal antibodies against MHCemb (BF-G6). By this procedure only the 55 kDa peptide of MHCemb is revealed, while the peptides from adult type MHC are negatives [compare in Fig. 2B lanes 8, 9 and 13 (MHCemb digests) with lanes 7 and 12 (MHCneo digests), and 6 and 11 (digests of MHC from EDL)]. The 55 kDa peptide of MHCemb is consistent with

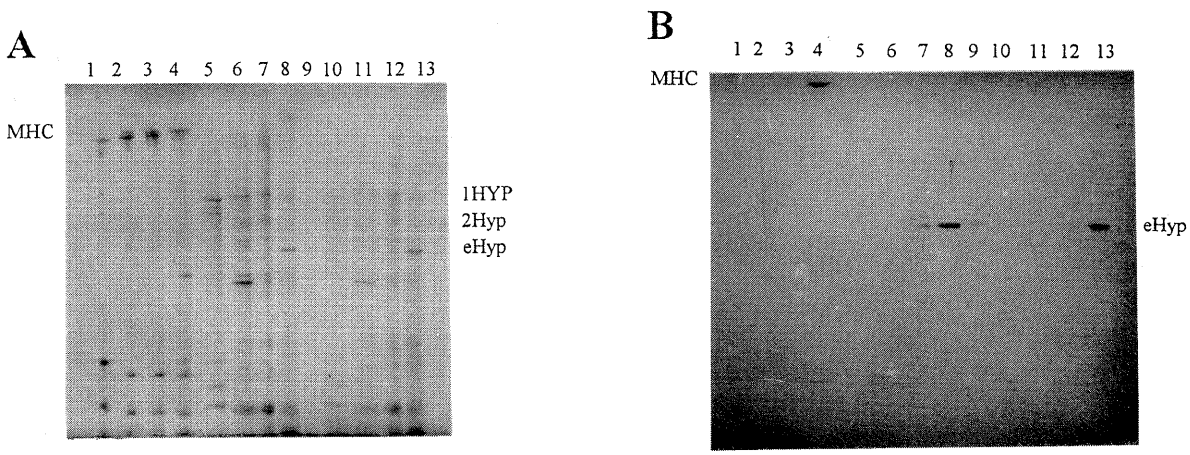
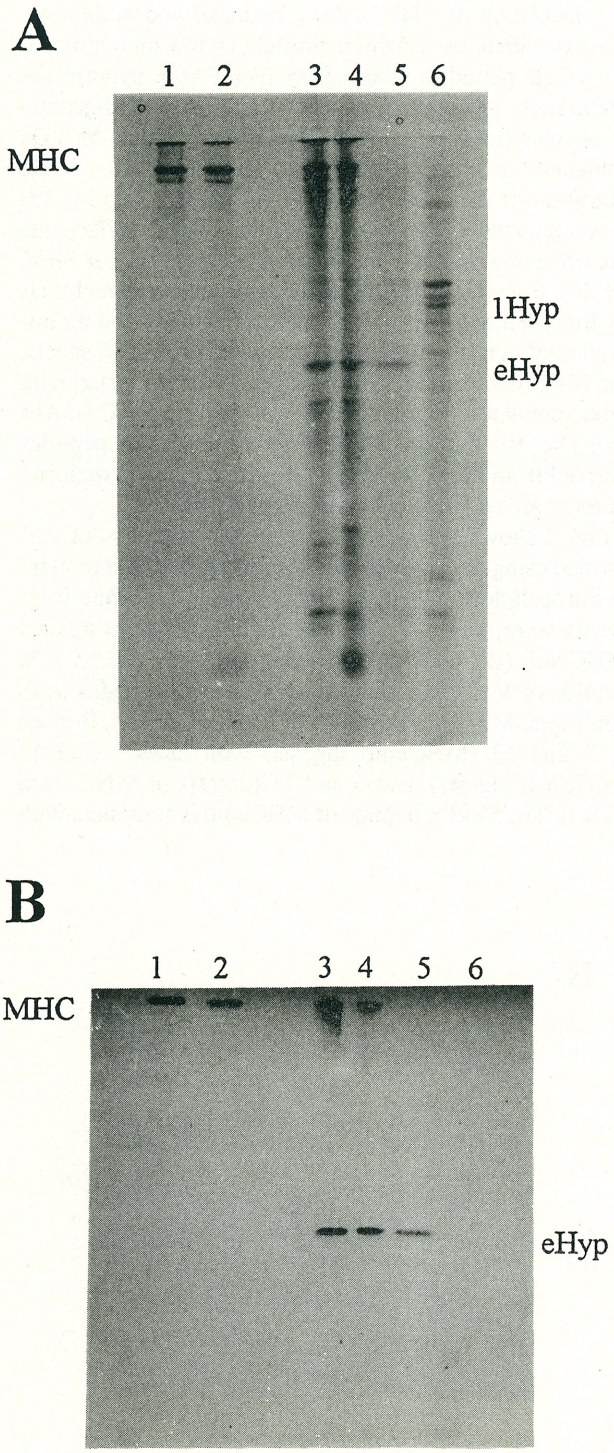


Figure 2. SDS PAGE and immunoblot analyses of hydroxylamine fragments of MHC from embryonic, neonatal, EDL and soleus rat muscles. A, Coomassie blue stain of SDS 10% PAGE analysis of MHC from: 5 µg of myosin purified from adult soleus (1), adult EDL (2), 7-day neonatal leg muscles (3), skeletal muscle of 18-day rat embryos (4); hydroxylamine-peptides of soleus myosin, 12 µg (5) and (10); hydroxylamine-peptides of EDL myosin 12 µg (6) and (11); hydroxylamine-peptides of neonatal myosin 12 µg (7) and (12); hydroxylamine-peptides of embryonic myosin 12 µg (8) and (13), and 0.6 µg (9); in samples (5) - (8), the reaction was stopped by dialysis, while in (10)-(13) TCA was used to block MHC digestion; B, Immunoblots of a slab identical to A reacted with BF-G6 an anti-MHCemb monoclonal Abs. MHC, myosin heavy chain; eHyP, hydroxylamine-peptide of MHCemb; 1HyP, hydroxylamine-peptide of MHC1; 2HyP, hydroxylamine-peptide of MHC2.



the aminoacid sequences deduced from MHC gene sequencing: MHCemb contains a glycine-asparagine peptide bond at position 583-584 [21]. Note that the neonatal myosin produces a peculiar pattern (Fig. 2, lane 7 and 12). The trace amounts of the 55 kDa peptide shown by immunoblotting in neonatal myosin digests (Fig 2B, lanes 7 and 12) is due to the low percentage of embryonic myosin known to be present in leg muscles of 7-day old rat puppies. Heterogeneity of muscle samples can be deduced from the relative proportion of hydroxylamine peptides in the digests: note that several high molecular weight peptides are present in the digest of myosin prepared from adult rat EDL, which is known to contain different proportion of at least four distict MHC [6].

Fig. 3 shows that a 55 kDa peptide, i.e. with an electrophoretic mobility identical to that of the rat embryonic myosin digest, is immunostained by the anti-MHCemb Abs in a hydroxylamine digest of human embryonic myosin purified from skeletal muscle of a 20-week foetus. As far as the hydroxylamine peptide is concerned, rat and human myosins are identical, so that allowing our method to be applied to human muscle pathology and physiopathology. The method allows to identify a 1-10% presence of embryonic myosin in an one µg sample of purified myosin (results not shown) and could be used to quantitate embryonic myosin in contractile proteins extracted from a few cryostat sections of biopsed muscle. The procedure is even more effective if after dyalisis against water an additional hour of dyalisis against 0.05% SDS, 96 mM glycine, 12.5 mM Tris, pH 8.2 is performed and then peptides are concentrated with KDS [19].

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Figure 3. SDS PAGE and immunoblot analyses of hydroxylamine fragments of embryonic MHC from human muscle. A, Coomassie blue stain of SDS 10% PAGE analysis of MHC from: 5 µg of myosin purified from skeletal muscle of 20-week human embryo (1) and 18-day rat embryo (2); 20 mg of hydroxylamine-peptides from contractile proteins of skeletal muscle of a 20-week human embryo (3) and of 18-day rat embryo (4); 12 µg of hydroxylamine-peptides of rat embryonic myosin (5) and of adult soleus myosin (6); B, Immunoblots of a slab identical to A reacted with BF-G6 an anti-MHCemb monoclonal Abs. MHC, myosin heavy chain; eHyP, hydroxylamine-peptide of MHCemb; 1HyP, hydroxylamine-peptide of MHC1.

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