Physiol. Res. 56: 659-662, 2007

RAPID COMMUNICATION

Mass Spectrometry Analyses of Rat 2b Myosin Heavy Chain Isoform

J. ŽURMANOVÁ[1,2], D. MALÁČOVÁ[1,2], F. PŮTA[2], P. NOVÁK[3], J. ŘÍČNÝ[1], T. SOUKUP[1]

[1]Institute of Physiology, Academy of Science of the Czech Republic, [2] Department of Physiology and Developmental Biology, Faculty of Science, Charles University, [3]Institute of Microbiology, Academy of Science of the Czech Republic, Prague, Czech Republic

Received May 4, 2007
Accepted June 21, 2007

Summary

We have separated 2b myosin heavy chain (MyHC) isoform from the rat extensor digitorum longus muscle by SDS-PAGE and analyzed it by two subsequent mass spectrometry techniques. After tryptic digestion, the obtained peptides were identified by Matrix-Assisted Laser Desorption/Ionisation reflectron Time of Flight mass spectrometry (MALDI-TOF MS) and sequenced by Liquid chromatography tandem mass spectrometry (ESI LC/MS/MS). The analyzed peptides proportionally covered 30 % of the 2b MyHC isoform sequence. The results suggest that the primary structure is identical with the highest probability to a NCBI database record ref|NP_062198.1|, representing the last updated record of rat 2b isoform. Nonetheless, four peptides carrying amino acid substitution(s) in comparison with the NCBI database record were identified.

Key words

Rat muscle myosin • Myosin heavy chain 2b isoform • MALDI TOF mass spectrometry • ESI LC/MS/MS mass spectrometry

Myosin heavy chain (MyHC) cDNA sequences are highly conserved among mammals including human, mouse and rat. Although the full sequences of human and mouse type 1, 2a, 2x/d and 2b MyHC isoforms have been described, the information for rat is still incomplete and often controversial, especially for the 2b isoform. When we started our experiments (2005), the NCBI database record contained only gi|34870888|ref|XP_340819.1|, annotated as "similar to MyHC 2b" and supposed to be a product of a splicing variant of Myh 4 gene. On the other hand, when we compared the primers used in RT-PCR studies of rat muscles (Mc Nally *et al*. 1989, DeNardi *et al*. 1993, Lieber *et al*. 1993, Jaschinski *et al*. 1998) with the available database information, we found that primers used for 2b isoform corresponded not only to the 2b-like isoform, but also to the sequence annotated as "catenin". In order to obtain more reliable data about the primary structure of MyHC 2b in the rat (useful for construction of new primers, as well), we have separated 2b MyHC isoform from the rat EDL muscle by SDS-PAGE (Fig.1) and analyzed it by two subsequent mass spectrometry techniques (MS).

Fax +420 241 062 164
E-mail: physres@biomed.cas.cz http://www.biomed.cas.cz/physiolres

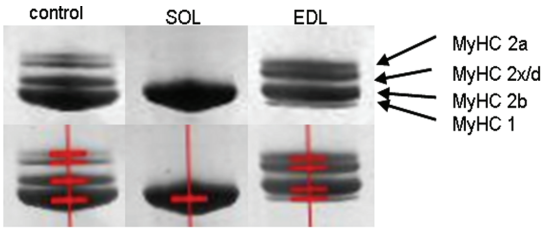

Fig. 1. SDS-PAGE separation of MyHC isoforms from 8-month-old female Lewis rat. SOL- soleus muscle, EDL- extensor digitorum longus muscle, control: SOL+EDL.

The separated 2b MyHC isoform bands (Fig.1) were stained by CBB-R 250 and stained protein bands were cut from the gel and washed several times with 10 mM dithiothreitol (DTT), 0.1 M 4-ethylmorpholine acetate (pH 8.1) in 50 % acetonitrile (MeCN). After the complete destaining, the gel was incubated with 60 mM iodoacetamide for 30 min at RT in dark, washed with water, shrunk by dehydration with MeCN and reswollen in water. Next, the gel was partly dried using a SpeedVac concentrator and then reconstituted with cleavage buffer containing 0.01 % 2-mercaptoethanol, 0.1 M 4-ethyl-morpholine acetate, 10 % MeCN and sequencing grade trypsin (Promega, 5 ng/µl). Proteolytic digestion was carried out overnight at 37 °C; the resulting peptides were extracted with 30 % MeCN/1 % formic acid and subjected to the two following MS analyses: **A)** Mass spectra were measured on a matrix-assisted laser desorption/ionisation reflectron time-of-flight MALDI-TOF mass spectrometer BIFLEX II (Bruker-Franzen, Bremen, Germany) equipped with a nitrogen laser (337 nm) and gridless delayed extraction ion source. Ion acceleration voltage was 19 kV and reflectron voltage was set to 20 kV. The spectra were calibrated externally using the monoisotopic $[M+H]^+$ ion of peptide standard somatostatin (Sigma). The saturated solution of α-cyano-4-hydroxy-cinnamic acid in 50 % ACN/0.2 % TFA was then used as a MALDI matrix. One µl of matrix solution was mixed with 1 µl of the sample on the target and the droplet was allowed to dry at ambient temperature. **B)** Tryptic peptide mixture was applied on the Magic-C18 column, 0.2x150 mm, 200 Å, 3 µm (Michrom Bioresources, CA) and separated using gradient elution: 10 min from 5 % MeCN/0.5 % acetic acid to 15 % MeCN/0.4 % acetic acid, 40 min from 15 % MeCN/ 0.5 % acetic acid to 40 % MeCN/0.4 % acetic acid and 10 min from 40 % MeCN/0.5 % acetic acid to 95 % MeCN/0.4 % acetic acid at flow rate 4 µl/min. The column was connected to a LCQ^{DECA} ion trap mass spectrometer (ThermoQuest, San Jose, CA) equipped

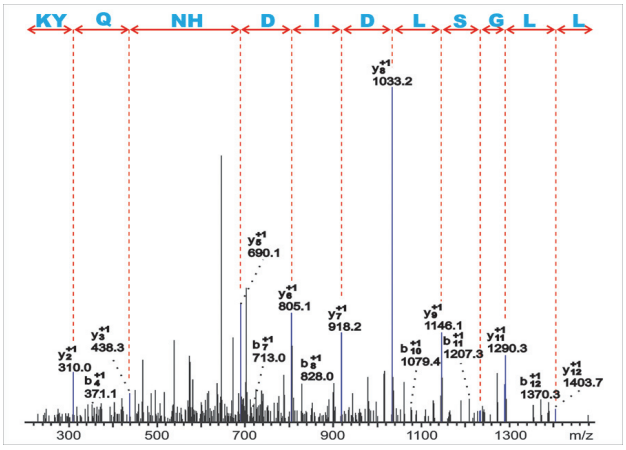

Fig. 2. The fragment spectrum of a tryptic peptide demonstrating the single amino acid mutation T>N of the rat 2b MyHC isoform sequence.

with a nanoelectrospray ion source (ESI LC/MS/MS). Spray voltage was held at 1.8 kV, tube lens voltage was –10 V. The heated capillary was kept at 150 °C with a voltage of 32 V. Collision energy was kept at 42 units and the activation time was 30 ms. Collisions were done from the first intense ion in each chromatographic peak and every 2 scans were accumulated. Positive-ion full scan and CID mass spectra were recorded. Full scan spectra were acquired over m/z range 350-1600 and selected spectra were interpreted by SEQUEST software in order to assign the peptide fragment spectra.

As example, the fragment spectrum of a tryptic peptide demonstrating the single amino acid mutation T>N in the rat 2b MyHC isoform sequence is shown in Figure 2. All peptides analyzed by MALDI-TOF and ESI LC/MS/MS (highlighted by colors in Fig. 3) were compared with database records of MyHC isoform variants using Multiple sequence alignment (CLUSTAL W 1.83, Higgins *et al.* 1994). The ref|NP_062198.1| was identified as the most likely variant of the rat 2b MyHC isoform. Both analyses have identified eight peptides specific for rat 2b MyHC (marked in red) and 35 peptides not specific only for the 2b MyHC isoform (marked in blue). They covered approximately 30 % of the 2b MyHC sequence. MALDI-TOF identified with high probability the presence of 19 peptides (non-underlined), four of them were 2b isoform specific (non-underlined red). ESI LC/MS/MS technique has confirmed the existence of 24 peptides (underlined), four of them specific to 2b isoform (underlined red). Furthermore, the latter technique has revealed presence of five peptides, whose primary structures differ from their database counterparts (Fig. 3 bottom, in green, the differing amino acids are

```
MSSDAEMAVFGEAAPYLRKSEKERIEAQNKPFDAKSSVFVVDAKESYVKATVQSREGGKV    60
TAKTEGGATVTVKEDQVFSMNPPKYDKIEDMAMMTHLHEPAVLYNLKERYAAWMIYTYSG   120
LFCVTVNPYKWLPVYNPEVVAAYRGKKRQEAPPHIFSISDNAYQFMLTDRENQSILITGE   180
SGAGKTVNTKRVIQYFATIAVTGDKKKEEAPSGKMQGTLEDQIISANPLLEAFGNAKTVR   240
NDNSSRFGKFIRIHFGATGKLASADIETYLLEKSRVTFQLKAERSYHIFYQVMSNKKPEL   300
IEMLLITTNPYDFAYVSQGEITVPSIDDQEELMATDTAVDILGFTADEKVAIYKLTGAVM   360
HYGNMKFKQKQREEQAEPDGTEVADKAAYLTSLNSADLLKALCYPRVKVGNEYVTKGQTV   420
QQVYNSVGALAKAMYEKMFLWMVTRINQQLDTKQPRQYFIGVLDIAGFEIFDFNTLEQLC   480
INFTNEKLQQFFNHHMFVLEQEEYKKEGIEWEFIDFGMDLAACIELIEKPMGIFSILEEE   540
CMFPKATDTSFKNKLYEQHLGKSNNFQKPKPAKGKAEAHFSLVHYAGTVDYNIIGWLDKN   600
KDPLNETVVGLYQKSGLKTLAFLFSGGQAAEAEGGGGKKGGKKKGSSFQTVSALFRENLN   660
KLMTNLKSTHPHFVRCLIPNETKTPGAMEHELVLHQLRCNGVLEGIRICRKGFPSRILYA   720
DFKQRYKVLNASAIPEGQFIDSKKASEKLLGSIDIDHTQYKFGHTKVFFKAGLLGTLEEM   780
RDEKLAQLITRTQAVCRGYLMRVEFRKMMERRESIFCIQYNVRAFMNVKHWPWMKLYFKI   840
KPLLKSAETEKEMATMKEDFEKAKEDLAKSEAKRKELEEKMVALMQEKNDLQLQVQAEAD   900
GLADAEERCDQLIKTKIQLEAKIKELTERAEDEEEINAELTAKKRKLEDECSELKKDIDD   960
LELTLAKVEKEKHATENKVKNLTEEMAGLDENIVKLTKEKKALQEAHQQTLDDLQAEEDK  1020
VNTLTKAKTKLEQQVDDLEGSLEQEKKLRMDLERAKRKLEGDLKLAQESTMDIENDKQQL  1080
DEKLKKKEFEMSNLQSKIEDEQALGMQLQKKIKELQARIEELEEEIEAERASRAKAEKQR  1140
SDLSRELEEISERLEEAGGATSAQIEMNKKREAEFQKMRRDLEEATLQHEATAAALRKKH  1200
ADSVAELGEQIDNLQRVKQKLEKEKSELKMEIDDLASNMETVSKAKGNLEKMCRTLEDQL  1260
SEVKTKEEEQQRLINELSAQKARLHTESGEFSRQLDEKDAMVSQLSRGKQAFTQQIEELK  1320
RQLEEESKAKNALAHALQSARHDCDLLREQYEEEQEAKAELQRAMSKANSEVAQWRTKYE  1380
TDAIQRTEELEEAKKKLAQRLQDAEEHVEAVNSKCASLEKTKQRLQNEVEDLMIDVERSN  1440
AACAALDKK QRNFDKVLAEWKQKYEETQAELEASQKESRSLSTELFKVKNAYEESLDQL  1500
ETLKRENKNLQQEISDLTEQIAEGGKHIHELEKIKKQIDQEKSELQASLEEAEASLEHEE  1560
GKILRIQLELNQVKSEIDRKIAEKDEEIDQLKRNHLRVVESMQSTLDAEIRSRNDALRIK  1620
KKMEGDLNEMEIQLNHANRQAAEAIRNLRNTQGMLKDTQLHLDDALRGQDDLKEQLAMVE  1680
RRANLMQAEIEELRASLEQTERSRRVAEQELLDASERVQLLHTQNTSLINTKKKLETDIS  1720
QIQGEMEDIVQEARNAEEKAKKAITDAAMMAEELKKEQDTSAHLERMKKNMEQTVKDLQH  1780
RLDEAEQLALKGGKKQIQKLEARVRELENEVENEQKRNIEAVKGLRKHERRVKELTYQTE  1840
EDRKNVLRLQDLVDKLQTKVKAYKRQAEEAEEQSNVNLAKFRKIQHELEEAEERADIAES  1900
QVNKLRVKSREVHTKVISEE
```

749	LLGSLDIDHNQYK	761
1181	DLEEATLQHEATAATLR	1197
1255	TLEDQLSEAR	1264
1401	LQDAEEAVEAVNAK	1414
1598	VVETMQSTLDAEIR	1611

Fig 3. The peptides obtained by trypsin cleavage of the rat EDL MyHC 2b isoform band and determined by MALDI-TOF MS analysis are highlighted in colors and those peptides sequenced by subsequent LCQ^{DECA} MS/MS analysis are underlined. These results are compared with database records of rat MyHC 2b isoform variants using Multiple sequence alignment (CLUSTAL W 1.83, Higgins *et al.* 1994). The analyzed peptides revealed that the primary structure is highly identical with the NCBI database record ref|NP_062198.1| for the rat 2b MyHC isoform. Peptides specific for rat MyHC 2b isoform are highlighted in red (**A**), peptides present also in other skeletal muscle MyHC isoforms are highlighted in blue (**A**), peptides with a point mutation according to (MS/MS) are shown in green (**A**) at the bottom (their database counterparts are marked by boxes), **X** - highlights point mutations revealed by ESI LC/MS/MS.

indicated by the yellow background).

MyHC 2b isoform is contained in 2B fibers which are the fastest from 2A, 2X/D and 2B fast fiber types in the rat hind limb skeletal muscles (for review see Schiaffino and Reggiani 1996). In the rat EDL muscle, 2B fibers represent the most frequent fiber type (Soukup *et al*. 2002, Zachařová *et al*. 2005, Vadászová-Soukup *et al*. 2006). Interestingly, in human muscles, the 2B fibers contain 2x/d MyHC isoform (Smerdu *et al.* 1994). Furthermore, the content of 2B fibers and of 2b MyHC isoform is increased in the EDL muscles of hyperthyroid rats (Vadászová *et al*. 2006a,b, for review see Soukup and Jirmanová 2000). The knowledge of the complete primary structure of rat 2b MyHC isoform could contribute to our understanding of physiological characteristics of this isoform. It could also be helpful for designing the most specific primers for (q)RT-PCR (Žurmanová *et al*. 2007). In conclusion, we have found eight peptides specific for 2b isoform by MALDI-TOF analysis, four of them have been proved by ESI LC/MS/MS technique, as well. Furthermore, ESI LC/MS/MS analysis has revealed five peptides

containing single amino acid substitution. Our data thus demonstrate that further detailed analysis is still necessary to describe the exact primary structure of the 2b MyHC isoform of the rat.

Acknowledgement

Supported by MYORES No. 511978, MSMT CR LC554, GACR 304/05/0327 and 305/06/1115 grants and by the Research projects AV0Z 50110509 and AV0Z 5020051.

References

DENARDI C, AUSONI S, MORETTI P, GORZA L, VELLECA M, BUCKINGHAM M, SCHIAFFINO S: Type 2X-myosin heavy chain is coded by a muscle fiber type-specific and developmentally regulated gene. *J Cell Biol* **123**: 823-835, 1993.

HIGGINS D, THOMPSON J, GIBSON T, THOMPSON JD, HIGGINS DG, GIBSON TJ, CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**: 4673-4680, 1994.

JASCHINSKI F, SCHULER M, PEUKER H, PETTE D: Changes in myosin heavy chain mRNA and protein isoforms of rat muscle during forced contractile activity. *Am J Physiol* **274:** C365-C370, 1998.

LIEBER RL, BODINE SC, BURKHOLDER TJ, PIEROTTI DJ, RYAN AF: Cloning and in situ hybridization of type-2A and type-2B rat skeletal muscle myosin tail region-implications for filament assembly. *Biochem Biophys Res Commun* **197**: 1312-1318, 1993.

MCNALLY EM, KRAFT R, BRAVO-ZEHNDER M, TAYLOR DA, LEINWAND LA: Full length rat alpha and beta cardiac myosin heavy chain sequences: comparisons suggest a molecular basis for functional differences. *J Mol Biol* **210**: 665-671, 1989.

SCHIAFFINO S, REGGIANI C: Molecular diversity of myofibrillar proteins: gene regulation and functional significance. *Physiol Rev* **76**: 371-423, 1996.

SMERDU V, KARSCH-MIZRACHI I, CAMPIONE M, LEINWAND L, SCHIAFFINO S: Type IIx myosin heavy chain transcripts are expressed in type IIb fibers of human skeletal muscle. *Am J Physiol* **267:** C1723-8, 1994.

SOUKUP T, JIRMANOVÁ I: Regulation of myosin expression in developing and regenerating extrafusal and intrafusal muscle fibres with special emphasis on the role of thyroid hormones. *Physiol Res* **49**: 617-633, 2000.

SOUKUP T, ZACHAŘOVÁ G, SMERDU V: Fibre type composition of soleus and extensor digitorum longus muscles in normal female inbred Lewis rats. *Acta Histochem* **104**: 399-405, 2002.

VADÁSZOVÁ A, HUDECOVÁ S, KRIŽANOVÁ O, SOUKUP T: Levels of myosin heavy chain mRNA transcripts and content of protein isoforms in the slow soleus muscle of 7-month-old rats with altered thyroid status. *Physiol Res* **55**: 221-225, 2006a.

VADÁSZOVÁ A, HUDECOVÁ S, KRIŽANOVÁ O, SOUKUP T: Levels of myosin heavy chain mRNA transcripts and protein isoforms in the fast extensor digitorum longus muscle of 7-month-old rats with chronic thyroid status alterations. *Physiol Res* **55**: 707-710, 2006b.

VADÁSZOVÁ-SOUKUP A, SMERDU V, ŽURMANOVÁ J, MALÁČOVÁ D, SOUKUP T: Unilateral foreign muscle transplantation has no effect on phenotype of unoperated rat muscles in long term experiments. *Physiol Res* **55**: 49P, 2006.

ZACHAŘOVÁ G, VADÁSZOVÁ A, SMERDU V, ASMUSSEN G, SOUKUP T: The effect of a unilateral muscle transplantation on the muscle fiber type and the MyHC isoform content in unoperated hind limb slow and fast muscles of the inbred Lewis rats. *Physiol Res* **54**: 691-696, 2005.

ŽURMANOVÁ J, MALÁČOVÁ D, PUTA F, ŘÍČNÝ J, SOUKUP T: Design of new primers for real time PCR of rat myosin heavy chain isoforms. *Physiol Res* **56**: 42P, 2007.

Corresponding author

Tomáš Soukup, Institute of Physiology AS CR, Vídeňská 1083, CZ-142 20 Prague, Czech Republic. Fax: +420 24106 2488. E-mail: tsoukup@biomed.cas.cz