Research in Veterinary Science xxx (2009) xxx–xxx

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

Establishment of a proteomic reference map for the *gastrocnemius* muscle in the rabbit (*Oryctolagus cuniculus*)

André M. Almeida [a,b,*], Alexandre Campos [b], Sofia van Harten [a], Luís Alfaro Cardoso [a], Ana Varela Coelho [b,c]

[a] *Instituto de Investigação Científica Tropical, Lisboa, Portugal & Centro Interdisciplinar de Investigação em Sanidade Animal, Lisboa, Portugal*
[b] *Instituto de Tecnologia Química e Biológica, Oeiras, Portugal*
[c] *Universidade de Évora, Évora, Portugal*

ARTICLE INFO

Article history:
Accepted 22 February 2009
Available online xxxx

Keywords:
Rabbit
Gastrocnemius
2D reference map

ABSTRACT

In several laboratory and production species, the establishment of a proteome reference map of a specific tissue has been accomplished. The rabbit is widely used as both a production and experimental animal. A lot of physiology research involving the *gastrocnemius* muscle of rabbit is described, although no reference proteome map is available. In this work, the first reference map of the rabbit's *gastrocnemius* muscle using 2D gel electrophoresis and the identification of proteins through peptide mass fingerprinting (PMF) was established. A total of 45 proteins were localized and identified with three major roles: cell structure and contractile apparatus; metabolic and cell defense proteins. A reference map of major proteins expressed is described enabling possible comparisons with other physiological studies.

The use of proteomics, the study of protein expression, is considered of major interest in 21st century research in animal science (Fadiel et al., 2005). Aiming to better understand proteome alterations, it is vital to have a reference proteome map for a specific tissue and species. Proteome maps of muscle have been described in laboratory species and are directed towards biomedicine. Regarding studies in farm animals, only domestic cattle (*Bos taurus*) seems to have proteome maps, specific to the *Longissimus thoracis* (Morzel et al., 2008) or *Semitendinosus* (Bouley et al., 2004; Chaze et al., 2006). Several other expression studies exist for pig and sheep.

The rabbit (*Oryctolagus cuniculus*) is an important species worldwide used as a laboratory animal and model for larger species. It is the basis of physiological, anatomical and disease models in humans and plays a major role as a meat-producing animal in Mediterranean Europe and parts of Asia. However, no muscle proteome map seems to be available in the literature. The aim of this study was to construct a reference proteome map for the *gastrocnemius* muscle of the rabbit, as this muscle is considered to be a model for muscle plasticity (Alford et al., 1987), is readily accessible (Almeida et al., 2006) and frequently used in muscle physiology studies. Proteome mapping will also provide a basis for studies on meat quality.

New Zealand white (NZR, *Oryctolagus cuniculus cuniculus*, $n = 4$) and Iberian wild (WR *Oryctolagus cuniculus algirus*, $n = 3$) rabbits were used at an age of 10–12 weeks. NZR weighed on average 3495.52 ± 67.67 g and WR 789.63 ± 49.61 g. European Union regulations on laboratory animal experimentation were followed. All animals were fed *ad libitum* on commercial pellets (Biona 701, Saprogal, Vila Chã de Ourique, Portugal, composition: 14.9% Crude Protein, 2.7% Crude Fat, 14% Crude Fibre and 13% Ash). Animals kept in individual cages with environmental enrichment, were allowed free access to water. After 30 days, animals were euthanized with 4% isoflurane (IsoFlo®, Veterinaria Esteve, Bologna, Italy). Muscle samples (*gastrocnemius*) were collected and snap-frozen in liquid nitrogen.

Total protein was extracted (Bouley et al., 2004) and quantified using PlusOne 2D Quant kit (GE Lifesciences, Uppsala, Sweden). Isoelectric focusing (IEF) followed by SDS–PAGE was used. One 2D gel was run per sample. 2D electrophoresis, strip equilibration and gel digitalization was conducted according to Görg et al. (2000) in the IPGphor system, using 24 cm strips with a linear pH gradient 3–10.

Gels were stained using colloidal Coomassie blue (Neuhoff et al., 1988) for 48 h and washed 3 times in double distilled water. Spot matching was conducted according to Soares et al. (2007) using Platinum software. Methodology for protein identification through PMF has been described (Lamy et al., 2008). Three replicates of each spots were excised and used for identification.

We constructed a reference map of the most representative proteins in the *gastrocnemius* muscle of the rabbit and identified as many proteins as possible. The identification map obtained in this study is presented in Fig. 1a. Most of the proteins present were found in all samples and spots appeared in the similar region,

* Corresponding author. Address: Instituto de Investigação Científica Tropical, Lisboa, Portugal & Centro Interdisciplinar de Investigação em Sanidade Animal, Lisboa, Portugal. Tel.: +35 1965461576; fax: +35 1213652869.
E-mail address: amalmeid@itqb.unl.pt (A.M. Almeida).

0034-5288/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.rvsc.2009.02.011

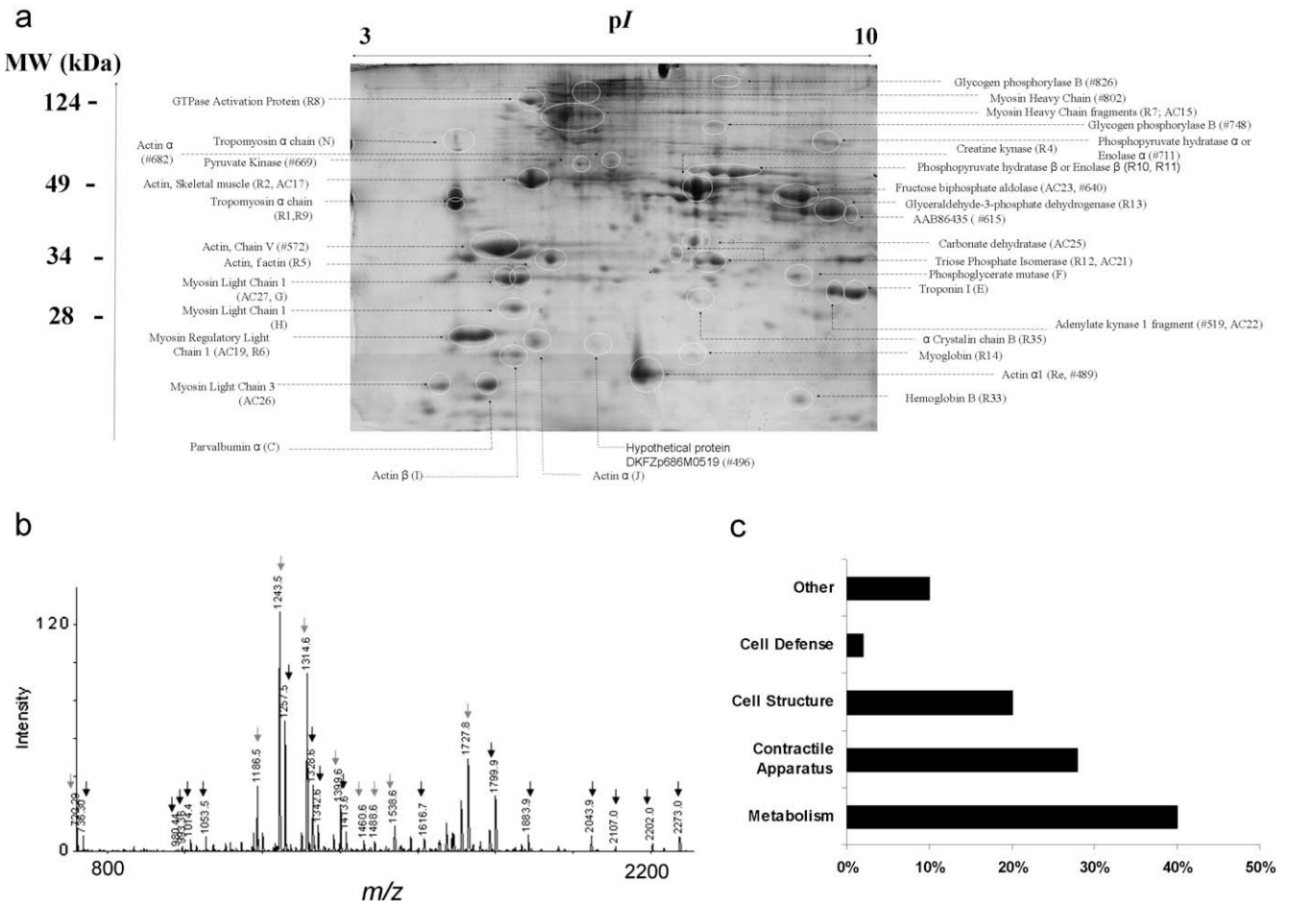

Fig. 1. Two dimensional reference map, mass spectra and distribution of proteins according role. a – Two dimensional reference map of wild rabbit *gastrocnemius* muscle. First dimension run was carried out in 24 cm pH 3–10 IPG strip and second dimension in a 12% SDS–PAGE gel, respectively, separating proteins according to their isoelectric point (pI) and the molecular mass (MW). Gels were stained with Coomassie Colloidal G250. Identified proteins are indicated with the most commonly used name. Indications in parenthesis concern reference numbers that can be found in Table 1. Molecular weight markers (MW) are presented on the left hand side. b – Mass Spectra obtained by Matrix-Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) technique for spots N. The monoisotopic mass of each individual peak considered is marked and used for protein identification by peptide mass fingerprinting using the Mascot program. Peaks submitted are marked with an arrow (Peptides Searched), black for unmatched and grey for matched peptides. As shown in Table 1, protein N was identified as Tropomysin α from rabbit. c – Distribution of proteins identified for the *gastrocnemius* muscle in the rabbit, classified according to general biochemical and physiological roles: metabolism; contractile apparatus, cell structure; cell defense and other.

demonstrating reproducibility of results. To the best of our knowledge, this is the first study attempting to map the proteome of the *gastrocnemius* muscle in the rabbit. Although a few skeletal muscle proteomes are characterized in cattle (Bouley et al., 2004), information on the mapping of the *gastrocnemius* muscle proteome in farm animals does not exist, although available for the laboratory rat (Gelfi et al., 2006). A comparison of the identification results obtained in this study and others available in the literature seem to show a consistency of patterns of results, particularly structural proteins.

The identification of proteins was accomplished through the analysis of spectra (Fig. 1b). Identified proteins were classified according to their role (Table 1, Fig. 1c). Results demonstrate that the majority of metabolic proteins are found in the alkaline region (pI 7–10), in contrast to cell structure and contractile apparatus proteins that were found in the pI 4–7 region.

We identified one protein involved in cell defense, α crystalin B chain. Although abundantly expressed (Kamradt et al., 2002), this protein is likely to have low levels of expression under control situations; hence difficult to detect during two-dimensional electrophoresis separation at pI ranges where the depletion of most abundant proteins is not conducted. The proportion of cell defense proteins identified by Bouley et al. (2004) is higher (16%) than the 2% obtained in our study. As those authors loaded 800 μg versus 600 μg in our study, results indicate that if higher amounts of protein are loaded, we are likely to obtain higher identification success.

Several metabolic proteins were identified, with special reference to glycolysis enzymes. Contractile apparatus and cell structure proteins identified included actin, myosin light, regulatory light and heavy chains and its fragments, tropomyosin α and β, and troponin I. Other proteins identified were parvalbumin, hemoglobin and myoglobin.

Despite the fact that identification was inconclusive for 20 spots, a total of 45 relevant spots were positively identified using the PMF methodology, a success rate of 70%. Protein identification through PMF was successful for abundant proteins and with relative success for low abundant proteins. However, identification was not fully achieved for proteins with lower molecular weights or located in the vicinity of abundant proteins due to a lower amount of peptides resulting from tryptic digests or possible spot overlapping. A strategy to overcome such difficulties would include 2D electrophoresis using different pH ranges, increasing the amount of loaded protein or conduct studies using immuno-

Table 1
Summary of protein spots identified by 2D gel and peptide mass fingerprinting technologies in rabbit *gastrocnemius* muscle. Each spot includes number of peptide matched per peptide search, estimated and theoretical molecular weight, coverage percentage, protein scores, MSDB database accession number and the protein manes including the species on which it is identified.

Reference	Peptide search/match	Estimated MW (kDa)	Theoretical MW/pI (Da/pI)	% coverage	Score	MSDB database accession number; protein name and species
Metabolism proteins						
R8	32/12	125	116657/6.2	18	89	S01966 GTPase-activating protein – bovine
#669	28/10	55	57305/7.7	16	70	1A49A pyruvate kinase (EC 2.7.1.40), chain A – rabbit
#826	65/17	200	92995/6.5	22	82	2GPN glycogen phosphorylase b (EC 2.4.1.1) – rabbit (fragment)
#748	18/10	90	96311/6.6	12	84	1NOJ glycogen phosphorylase (EC 2.4.1.1) – rabbit
#711	52/11	55	47428/6.2	26	79	A23126 phosphopyruvate hydratase (EC 4.2.1.11) or alpha-enolase (EC 4.2.1.11)
R4	46/12	40	43313/6.6	35	90	KIRBCM creatine kinase (EC 2.7.3.2) chain M – rabbit
R10	34/11	49	47174/8.1	31	97	A37210 phosphopyruvate hydratase (EC 4.2.1.11) beta – or beta-enolase
R11	50/11	49	47174/8.1	30	71	A37210 phosphopyruvate hydratase (EC 4.2.1.11) beta – rabbit or beta-enolase
AC23	51/13	38	39774/8.3	44	96	ADRBA fructose-bisphosphate aldolase (EC 4.1.2.13) A – rabbit
#640	64/13	38	39643/8.4	28	65	ADRBA fructose-bisphosphate aldolase (EC 4.1.2.13) A – rabbit
R13	52/15	37	35914/6.9	34	84	DEPGG3 glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) – pig
#615	16/8	36	29991/ 8.3	31	110	AAB86435 glyceraldehyde-3-phosphate dehydrogenase: – sheep
AC25	49/8	35	29647/7.8	26	67	A22612 carbonate dehydratase (EC 4.2.1.1) III – horse
R12	44/9	30	26894/7.1	41	82	ISRBT triose-phosphate isomerase (EC 5.3.1.1) – rabbit
AC21	17/6	30	26894/7.1	22	72	ISRBT triose-phosphate isomerase (EC 5.3.1.1) – rabbit
F	55/14	31	28788/9.0	36	98	PGAM2_HUMAN Phosphoglycerate mutase 2 (EC 5.4.2.1) (EC 5.4.2.4) (EC 3.1.3.13) human
AC22	13/5	28	21738/8.9	30	61	KIRBA adenylate kinase (EC 2.7.4.3) – rabbit
#519	30/10	28	21719/8.9	40	94	Q53EY8_HUMAN Adenylate kinase 1 variant (fragment) human
Contractile apparatus proteins						
N	29/9	70	32718/4.7	20	80	TMRBA tropomyosin alpha chain, cardiac and skeletal muscle – rabbit
R1	42/9	45	32931/4.7	20	62	TMRBB tropomyosin beta chain, skeletal muscle – rabbit
R9	57/15	45	32718/4.7	35	112	TMRBA tropomyosin alpha chain, cardiac and skeletal muscle – rabbit
AC27	29/7	30	20862/4.9	39	75	MLE1_RABIT Myosin light chain 1, skeletal muscle isoform – rabbit
G	45/8	25	20862/4.9	42	77	MLE1_RABIT Myosin light chain 1, skeletal muscle isoform –rabbit
H	38/7	25	20862/4.9	38	70	MLE1_RABIT Myosin light chain 1, skeletal muscle isoform – rabbit
AC19	19/9	20	18997/4.8	44	107	MLRS_RABIT Myosin regulatory light chain 2, skeletal muscle isoform type 2 – rabbit
R6	39/11	20	18997/4.8	29	89	MLRS_RABIT Myosin regulatory light chain 2, skeletal muscle isoform type 2 – rabbit
AC26	31/6	17	16573/4.6	44	64	MLE3_RABIT Myosin light chain 3, skeletal muscle – rabbit
R7	102/26	125	78732/5.5	35	92	S00084 myosin heavy chain, fast skeletal muscle – rabbit (fragment)
AC15	58/16	115	103804/5.3	23	77	Q63939_9MURI Myosin heavy chain (fragment) – *Rattus* sp.
#802	55/33	200	223841/5.6	21	167	A59293 skeletal myosin heavy chain – rabbit
E	40/10	29	21241/8.9	46	79	TNNI2_RABIT Troponin I, fast skeletal muscle – rabbit
Cell structure proteins						
#682	27/10	60	42075/5.2	18	91	1DB0A actin, chain A – rabbit (fragment)
R2	38/12	49	42090/5.2	33	118	ATBOSM actin, aortic smooth muscle – bovine
AC17	25/8	49	41640/5.2	22	76	1ALMV actin f-actin, chain V – rabbit
#572	28/13	40	41640/5.2	25	109	1ALMV actin f-actin, chain V – rabbit
R5	38/13	34	41640/5.2	36	106	1ALMV actin f-actin, chain V – rabbit
I	42/9	29	42052/5.3	28	71	ATRBB actin beta, non-muscle – rabbit
J	66/11	29	42366/5.2	28	76	A24904 actin alpha, skeletal muscle – mouse
R3	59/11	15	42366/5.2	23	60	A24904 actin alpha, skeletal muscle – mouse
#489	42/9	15	41640/5.2	18	63	1ALMV actin f-actin, chain V – rabbit
Cell defense proteins						
R35	40/9	28	20094/6.8	37	87	A53871 alpha-crystalline chain B – rabbit
Other proteins						
C	11/6	15	11926/5.1	40	77	PVRB parvalbumin alpha – rabbit
#496	13/5	25	22986/10.6	35	68	Q63HP1_HUMAN Hypothetical protein DKFZp686M0519.– *Homo sapiens* (human)
R14	32/8	20	17079/6.7	49	87	MYRB myoglobin – rabbit
R33	51/9	15	16048/8.1	75	100	HBB_RABIT Hemoglobin subunit beta-1/2 rabbit

MW – molecular weight; pI – isoelectric point; score is $-10 * \mathrm{Log}(P)$ and P is the probability that the observed match is a random event and protein scores greater than 59 are significant when $p < 0.05$.

depletion. Such strategies, if using PMF, would still depend on the characterization of rabbit muscle proteins or their homology to other species' proteins. If the similarity would prove to be low, it would be advisable to use MS/MS or *de novo* sequencing approaches.

This is the first time that the proteome of the *gastrocnemius* muscle is described for the rabbit. Although several proteins failed to be identified, positive identification was accomplished for most of the proteins. Data presented in this study provides researchers with a reference to which results can be compared.

Please cite this article in press as: Almeida, A.M., et al. Establishment of a proteomic reference map for the *gastrocnemius* ... Res. Vet. Sci. (2009), doi:10.1016/j.rvsc.2009.02.011

Acknowledgements

The authors are grateful for the financial support from *Fundação para a Ciência e a Tecnologia* (grants SFRH/BD/4943/2001 – S. van Harten and SFRH/BPD/20783/2004 – A. Campos). The support of the Faculty of Veterinary Medicine of Lisbon is also acknowledged. The collaborations of Gonçalo Costa (ITQB, Portugal) in fruitful discussions and Tanya Kilminster (DAFWA, Merredin, Australia) for English editing are appreciated.

References

Alford, E.K., Roy, R.R., Hodgson, J.A., Edgerton, V.R., 1987. Electromyography of rat soleus, medial gastrocnemius and tibialis anterior during high limb suspension. Experimental Neurology 96, 635–649.

Almeida, A.M., Van Harten, S., Cardoso, L.A., 2006. Myofibrillar protein status of the gastrocnemius in male rats, effect of mild undernutrition. Scandinavian Journal of Laboratory Animal Science 33, 101–106.

Bouley, J., Chambon, C., Picard, B., 2004. Mapping of bovine skeletal muscle proteins using two-dimensional gel electrophoresis and mass spectrometry. Proteomics 4, 1811–1824.

Chaze, T., Bouley, J., Chambon, C., Bsrboiron, C., Piccard, B., 2006. Mapping of alkaline proteins in bovine skeletal muscle. Proteomics 6, 2571–2575.

Fadiel, A., Anidi, I., Eichenbaum, D., 2005. Farm animal genetics and informatics, an update. Nucleic Acids Research 33, 6308–6318.

Gelfi, C., Viganò, A., De Palma, S., Ripamonti, M., Begum, S., Cerreteli, P., Wait, R., 2006. 2-D protein maps of rat *gastrocnemius* and *soleus* muscles, a tool for muscle plasticity assessment. Proteomics 6, 321–340.

Görg, A., Obermaier, C., Boguth, G., Harder, A., Scheibe, B., Wildgruber, R., Weiss, W., 2000. The current state of two-dimensional electrophoresis with immobilized pH gradients. Electrophoresis 21, 1037–1053.

Kamradt, M.C., Cheng, F., Sam, S., Cryns, V.L., 2002. The small heat shock protein αB-crystalline negatively regulates apoptosis during myogenic differentiation by inhibiting caspase-3 activation. Journal of Biological Chemistry 277, 38731–38736.

Lamy, E., da Costa, G., Capela e Silva, F., Potes, J., Coelho, A.V., Baptista, E.S., 2008. Comparison of electrophoretic protein profiles from sheep and goat parotid saliva. Journal of Chemical Ecology 34, 388–397.

Morzel, M., Terlow, C., Chambon, C., Micol, D., Piccard, B., 2008. Muscle proteome and meat qualities of *Longissimus thoracis* of "Blonde d'Aquitaine" young bulls, a central role of HSP27 isoforms. Meat Science 78, 297–304.

Neuhoff, V., Arold, N., Taube, D., Ehrhardt, W., 1988. Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. Electrophoresis 9, 255–262.

Soares, N.C., Francisco, R., Ricardo, C.P., Jackson, P.A., 2007. Proteomics of ionically bound and soluble extracellular proteins in *Medicago truncatula* leaves. Proteomics 7, 2070–2082.

Please cite this article in press as: Almeida, A.M., et al. Establishment of a proteomic reference map for the *gastrocnemius* ... Res. Vet. Sci. (2009), doi:10.1016/j.rvsc.2009.02.011