Journal of Chromatography B, 878 (2010) 3370–3374

Contents lists available at ScienceDirect

Journal of Chromatography B

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

HPLC–MS/MS shotgun proteomic research of deer antlers with multiparallel protein extraction methods

Liang Gao[a,b,1], Dingyin Tao[a,b], Yichu Shan[a], Zhen Liang[a], Lihua Zhang[a,*], Yushu Huo[a], Yukui Zhang[a]

[a] Key Laboratory of Separation Science for Analytical Chemistry, National Chromatographic Research & Analysis Center, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116023, China
[b] Graduate School of the Chinese Academy of Sciences, Beijing 100039, China

ARTICLE INFO

Article history:
Received 12 July 2010
Accepted 24 October 2010
Available online 30 October 2010

Keywords:
Deer antler
μRPLC–ESI-MS/MS
Parallel protein extraction
Shotgun proteomic research
Serially coupled microcolumn

ABSTRACT

Deer antlers mature rapidly in 60 days, and subsequently shed in 5 days with rapid ossification. During this procedure, the function of deer antlers changes significantly. Therefore, the profiling of antler proteome is helpful to discover important growing and shedding regulation proteins, which might be of great significance for studying development and regeneration. In this study, a parallel protein extraction strategy was developed to extract proteins from antlers of red deer with five different lysis solutions, followed by shotgun proteomic analysis by microflow reversed-phase liquid chromatography/electrospray ionization/tandem mass spectrometry (μRPLC–ESI-MS/MS) with a 30 cm-long serially coupled microcolumn. Our experimental results showed that the identified proteins extracted by five kinds of lysis solution were complementary to each other. In total, 416 unique proteins were identified, with relative molecular masses from 2000 to 600,000, and isoelectric points from 3.84 to 11.57. All these results demonstrate that the combination of parallel protein extraction strategy and μRPLC–ESI-MS/MS analysis with serially coupled long microcolumns might be of great significance for comprehensive proteomic research of deer antler.

1. Introduction

Deer antlers mature rapidly in 60 days, and subsequently shed in 5 days. This procedure is co-regulated by various kinds of small and large biomolecules, so antler has been regarded as good model to study the regeneration of nerves, vessels, cartilages, skins and bones [1–5]. Several kinds of factors including retinoic acid, estrogen receptor, androgen hormones and various growth factors have been reported to affect the growth and differentiation of antlers [6–10]. During the rapid developmental procedure of antler, the proteins which are essential for all life processes of antler change significantly. Therefore, the profiling of deer antler proteome is vital for understanding the self-differentiation capacity of antlers.

However, until now, only one paper was published to study the proteome of deer antler [11], in which proteins extracted by 9 M urea with 4% (m/v) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS), 35 mM Tris(hydroxymethyl)aminomethane (Tris) and 65 mM dithiothreitol (DTT) were further separated by two-dimensional electrophoresis (2-DE), followed by in-gel digestion and protein identification by matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (MALDI-TOF MS). In total, 130 proteins were identified by searching the results from public database of mammalian and self-programmed draft of antler cDNA database. However, according to the genome of antler, the identified protein number is far less than expected. Therefore, to obtain comprehensive proteomic information of deer antlers, both protein extraction strategy and identification method should be further improved.

Efficient protein extraction from tissues, especially from ossified tissues, is one of the most critical issues for proteomic research. Since deer antler is partially ossified cornu, to a certain extent, the protein extraction method might be similar to that for bones. In previous research, bone proteins were often extracted with a single lysis solution, and the extraction efficiency was limited [12,13]. Recently, Jiang et al. developed a sequential protein extraction protocol to obtain proteins from dog bones. Using a shotgun strategy based on two-dimensional liquid chromatography/tandem mass spectrometry (2D-LC–MS/MS) analysis, 2479 unique proteins were identified [14]. However, the sequential extraction might lead to the distribution of low-abundance proteins simultaneously in several extracts, resulting in decreased protein identification capacity.

Although 2-DE is a powerful tool for proteome separation [15,16], it is of limited use in the analysis of extremely basic or acidic, small or large, and hydrophobic proteins. Recently, these limitations could be solved by 2D-LC–MS/MS analysis

* Corresponding author. Tel.: +86 411 84379720; fax: +86 411 84379560.
E-mail address: lihuazhang@dicp.ac.cn (L. Zhang).
[1] The author made the same contribution to this work.

1570-0232/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2010.10.022

[17,18]. However, the analysis time is usually long, and the reproducibility should be further improved. In our recent work, microflow reversed-phase liquid chromatography (μRPLC) with a serially coupled column was developed for proteome analysis, by which the time-based protein identification efficiency could reach 249 proteins *per* hour [19], higher than that obtained with other strategies, such as nanoflow liquid chromatography/tandem mass spectrometry [20], multidimensional protein identification technology (MudPIT) [18] and dual trap-based two-dimensional nanoflow liquid chromatography/tandem mass spectrometry [21].

In this work, to improve the protein extraction efficiency from deer antler, five kinds of extraction solutions were used in parallel, including 1.2 M HCl, 2 M NaOH, 100 mM Tris + 6 M guanidine-HCl, 100 mM Tris + 6 M guanidine-HCl + 0.5 M ethylene diamine tetraacetic acid (EDTA), and 9 M urea + 4% (m/v) CHAPS + 35 mM Tris + 65 mM DTT. In addition, to improve the separation and identification capacity of proteins, the tryptic digests of proteins extracted from each kind of solution were further analyzed by μRPLC-ESI-MS/MS with a serially coupled long microcolumn. Using such strategies, comprehensive proteome information of antler could be obtained, and several proteins were found closely related to the growth and ossification of deer antler.

2. Materials and methods

2.1. Chemicals and materials

Frits (1.59 mm diameter, 0.76 mm thickness, 2 μm pore) and PEEKsil (0.3 mm i.d.) tubes were purchased from Upchurch Scientific (Oak Harbor, WA, USA). Protease inhibitors cocktail and acetonitrile (ACN) (HPLC grade) were ordered from Merck (Darmstadt, Germany). Formic acid was bought from Kermel (Tianjin, China). Urea was purchased from Invitrogen (Carosbad, CA, USA). DTT and iodoacetamide (IAA) were from Acros (Morris plains, NJ, USA). TPCK treated trypsin (bovine pancreas) and Tris were from Sigma (St. Louis, MO, USA). C_{18} silica particles (5 μm, 300 Å pore) were bought from Agela Technologies (Tianjin, China). Syringe filters (0.45 μm × 25 mm) were ordered from Millipore (Bedford, MA, USA). Water was purified by a Milli-Q system. All other chemicals and solvents were analytical-grade.

2.2. Sample preparation

Antlers were collected from a 5-year-old male red deer (*Cervus elaphus*), 15 days after the antler generated from the pedicles. The antlers were removed by cutting the proximal region with a surgical hand-saw and frozen dried after blood removal with a vacuum pump. The growing tip was ground to powder in liquid nitrogen for further study.

One gram of powder was respectively incubated with 4 mL of each extract solution at 4 °C for 24 h, including 1.2 M HCl, 2 M NaOH, 100 mM Tris + 6 M guanidine-HCl, 100 mM Tris + 6 M guanidine-HCl + 0.5 M EDTA, and 9 M urea + 4% (m/v) CHAPS + 35 mM Tris + 65 mM DTT, followed by the addition of 1/100 (v/v) protease inhibitors cocktail, which were further marked as Extracts 1, 2, 3, 4 and 5. After centrifugation at 3866 × *g* for 0.5 h, the supernatant of Extracts 1–5 was filtered, precipitated with acetone (acetone/sample = 4/1, v/v) at −20 °C overnight and after removal of the supernatant precipitated protein was dissolved in the lysis solution containing 100 mM Tris with 6 M guanidine-HCl. Protein concentrations were determined by Bradford assay [22] using bovine serum albumin (BSA) as a standard protein. Extracted proteins were further reduced with DTT and alkylated with IAA. Then, the samples were diluted 6 times with 50 mM Tris–HCl, and the pH values were adjusted to 8.3. Finally, the proteins were digested with trypsin for 24 h at 37 °C using an enzyme-to-substrate ratio of 1:50 (m/m).

2.3. μRPLC–MS/MS analysis

The digests of each kind of extracted proteins were analyzed by μRPLC–ESI-MS/MS with a serially coupled microcolumn, performed on a paradigm GM4 μHPLC system (Michrom Bioresources Inc., Auburn, CA, USA) coupled with an LCQ^{DUO} quadrupole ion trap mass spectrometer (Thermo Fisher, San Jose, CA, USA). Twenty-five micrograms of deer antler protein digests was loaded onto a self-packed C_{18} trap column (40 mm × 0.5 mm i.d.) for on-line desalting, followed by separation with a 30 cm-long microcolumn (0.3 mm i.d.), which was serially connected by three self-packed C_{18} microcolumns, with the length respectively as 5, 10 and 15 cm. The binary mobile phase consisted of solvents A (0.1% HCOOH + 98% H_2O + 2% ACN, v/v) and B (0.1% HCOOH + 98% ACN + 2% H_2O, v/v). The flow rate was set at 6 μL/min. The peptides separation was performed with a mobile-phase gradient as follows: 0–10 min, 0% B; 10–360 min, 0–40% B; 360–380 min, 80% B.

For ESI-MS/MS, the spray voltage was set at 2.0 kV, and the normalized collision energy was set at 35.0%. The temperature of the ion-transfer capillary was set at 150 °C. The scanning range of relative molecular mass was set at 400–2000. The scan time was set at 380 min. System control and data collection were completed by Xcalibur software version 1.4 (Thermo Fisher, San Jose, CA, USA).

2.4. Data analysis

Data analysis was performed based on the cumulative total proteins identified in three replicative runs. The collected MS data files were converted to the MASCOT generic format (mgf). The combined mgf-files of the three replicative runs were sent to a MASCOT server (Version 2.2.04, Matrix Science) for automated peptide identification using the Swiss-Prot/UniProt database (www.uniprot.org/downloads, 2010-6-2 version) with mammalian taxonomy restriction, since current public database of deer contained limited entries of protein sequences (6920 entries in the NCBI database). The following Mascot settings were used: carbamidomethyl was specified as a fixed modification; methionine oxidation was specified as variable modifications. Two missed cleavage was allowed. The precursor mass tolerance was set to 2.0 Da and the fragment mass tolerance to 1.0 Da. The significance threshold was set to $p < 0.05$. The ion score cut-off was set to 20.

3. Results and discussion

3.1. Protein extraction by various solutions

To extract proteins from deer antler, various kinds of lysis solution were applied. HCl (1.2 M) and NaOH (2 M) were employed to extract acidic and basic proteins respectively. The third lysis solution, composed of 100 mM Tris and 6 M guanidine-HCl, was commonly used for proteome research to extract aqueous-soluble proteins. The fourth lysis solution, containing 100 mM Tris, 6 M guanidine-HCl and 0.5 M EDTA, was used to extract noncollagenous proteins closely associated with hydroxyapatite crystallites in the bone matrix [23]. The fifth lysis solution, composed of 9 M urea, 4% (m/v) CHAPS, 35 mM Tris and 65 mM DTT, was adapted from Ref. [11] for further comparison. As shown in Table 1, except for 1.2 M HCl, a large amount of protein was extracted with 2 M NaOH, 100 mM Tris + 6 M guanidine-HCl, 100 mM Tris + 6 M guanidine-HCl + 0.5 M EDTA, as well as 9 M

Table 1
Protein concentrations and cumulative number of proteins identified in three replicative injections of Extracts 1–5.

Sample name	Lysis solution	Protein concentration (mg/mL)	Number of identified proteins
Extract 1	1.2 M HCl	1.35	153
Extract 2	2 M NaOH	17.54	49
Extract 3	100 mM Tris + 6 M guanidine-HCl	21.41	161
Extract 4	100 mM Tris + 6 M guanidine-HCl + 0.5 M EDTA	26.95	153
Extract 5	9 M urea + 4% (w/v) CHAPS + 35 mM Tris + 65 mM DTT	29.14	138

urea + 4% (m/v) CHAPS + 35 mM Tris + 65 mM DTT as lysis solution.

3.2. Protein identification by μRPLC–ESI-MS/MS

The digests of extracted proteins were further analyzed by μRPLC–ESI-MS/MS with a serially coupled microcolumn, and the base peak chromatograms were shown in Supporting Fig. 1. Protein concentrations of Extracts 1–5 as well as cumulative number of proteins identified in three replicative injections are shown in Table 1. Although the extracted protein concentration by HCl is low, the number of identified proteins is as high as those obtained by the other lysis solutions. For proteins extracted by NaOH, although the protein concentration is high, only 49 unique proteins were identified. However, the identification of low abundant proteins might be hindered due to the presence of several high-abundance proteins, such as keratin and hemoglobin. In addition, the identified protein number from Extract 5 was similar to the previous report [11]. However, by further comparison, only 28 proteins were the same, which indicated that proteins identified by μRPLC–ESI-MS/MS were complementary to those found by 2-DE combined with MALDI-TOF MS. With our parallel extraction strategy, 416 unique proteins could be identified from deer antler (shown in Supporting Table 1).

The M_rs and p*I*s of the identified proteins could be obtained directly by database searching with MASCOT. The distribution of relative molecular masses (M_r) and isoelectric points (p*I*) of proteins identified by various extraction methods were shown in Fig. 1. Their M_r and p*I* range from 2000 to 600,000 and from 3.84 to 11.57 respectively, which were obviously broader than those obtained in previously work (M_r 3800–140,000 and p*I* 3.2–9.76) [11]. It showed that the combination of parallel protein extraction strategy and μRPLC–ESI-MS/MS analysis with a serially coupled column was powerful for antler proteomic research.

In addition, it could be seen that the M_rs of most identified proteins from deer antler (93.3%) were in the range from 10,000 to 200,000, and the p*I*s were mainly distributed in ranges of 5.0–7.0 (43.0%) and 8.0–10.0 (27.6%).

3.3. Complementarity of various extraction methods

To avoid the effect of the low scan rate of LCQ on the identification of proteins from deer antler, three replicative runs of μRPLC–ESI-MS/MS for each sample were performed, and the cumulative results were used for the comparison of different extraction methods. As shown in Fig. 2, among 416 identified proteins, only 11 proteins (2.64%) were found in all extracts, indicating a low overlapping ratio. Oppositely, 78, 29, 61, 61 and 59 proteins were respectively observed in each protein extract, accounting for about 69.2% of all identified proteins. In addition, the overlapping ratios of different extraction methods were rather low. All these results demonstrate the good complementarity of five extraction methods, which should contribute to the obvious improvement on identified protein number and more comprehensive information about antler proteome.

3.4. Deer antler proteome analysis

Although the blood was removed with a vacuum pump, several serum-derived proteins including serum albumin, hemoglobin and alpha-2-HS-glycoprotein were identified because they bind to the bone hydroxyapatite crystals in the mineral compartment and could not be removed completely.

The identified proteins from deer antler were characterized from the aspects of subcellular locations and involved biological processes with GoMiner program [24].

Totally, 256 identified proteins (61.5%) were annotated in subcellular location, and distributed in almost every cell compartment, as shown in Fig. 3. The majority, 87 proteins (34.0%), were membrane proteins, which might play important roles in vital cellular processes and signaling pathways. The proteomic research of membrane proteins still presents great challenges due to the high hydrophobicity which resulted in the difficulties in extraction, solubilization, digestion and separation. Among identified membrane proteins, 31 proteins (35.6%) were found in Extract 1, which might indicate good solubility of acidic buffers for membrane proteins.

Furthermore, 235 identified proteins (56.5%) were annotated involving in biological process as shown in Fig. 4. Fourteen of these proteins involved in biological process were related to growth, including chondroadherin, superoxide dismutase [Cu–Zn], ubiquitin, 60S ribosomal protein L30, macrophage migration inhibitory factor, nucleophosmin, tuberin, fibrinogen alpha chain, gelsolin, chromodomain-helicase-DNA-binding protein 7, actin, alpha-enolase, beta-enolase, breast cancer type 2 susceptibility protein homolog. Among these proteins related to growth, alpha-enolase and tuberin negatively regulate cell growth, while macrophage migration inhibitory factor and chromodomain-helicase-DNA-binding protein 7 positively regulate axon regeneration and multicellular organism growth, respectively. That such proteins simultaneously exist in deer antler demonstrates the complex mechanism of growth of antler. Further study of comparative proteome research of antler in different stages should be performed to better understand the growth of antler.

In addition, 7 of these identified proteins, including osteomodulin, osteopontin, osteonectin, exostosin-2, calcitonin receptor, neurofibromin and fibronectin, were engaged in ossification, which plays an important role in the development procedure of antler. Osteomodulin has a function in binding of osteoblasts via the alpha(V)beta(3)-integrin, and may be implicated in biomineralization processes [25]. Osteopontin binds tightly to hydroxyapatite and appears to form an integral part of the mineralized matrix [26]. Osteonectin appears to regulate cell growth through interactions with the extracellular matrix and cytokines [27]. Other proteins produced at different stages of osteoblast maturation, such as exostosin-2, calcitonin receptor, neurofibromin and fibronectin, were also identified. The identification of such proteins related to growth and ossification demonstrated the effectivity of the developed strategy for antler proteome research.

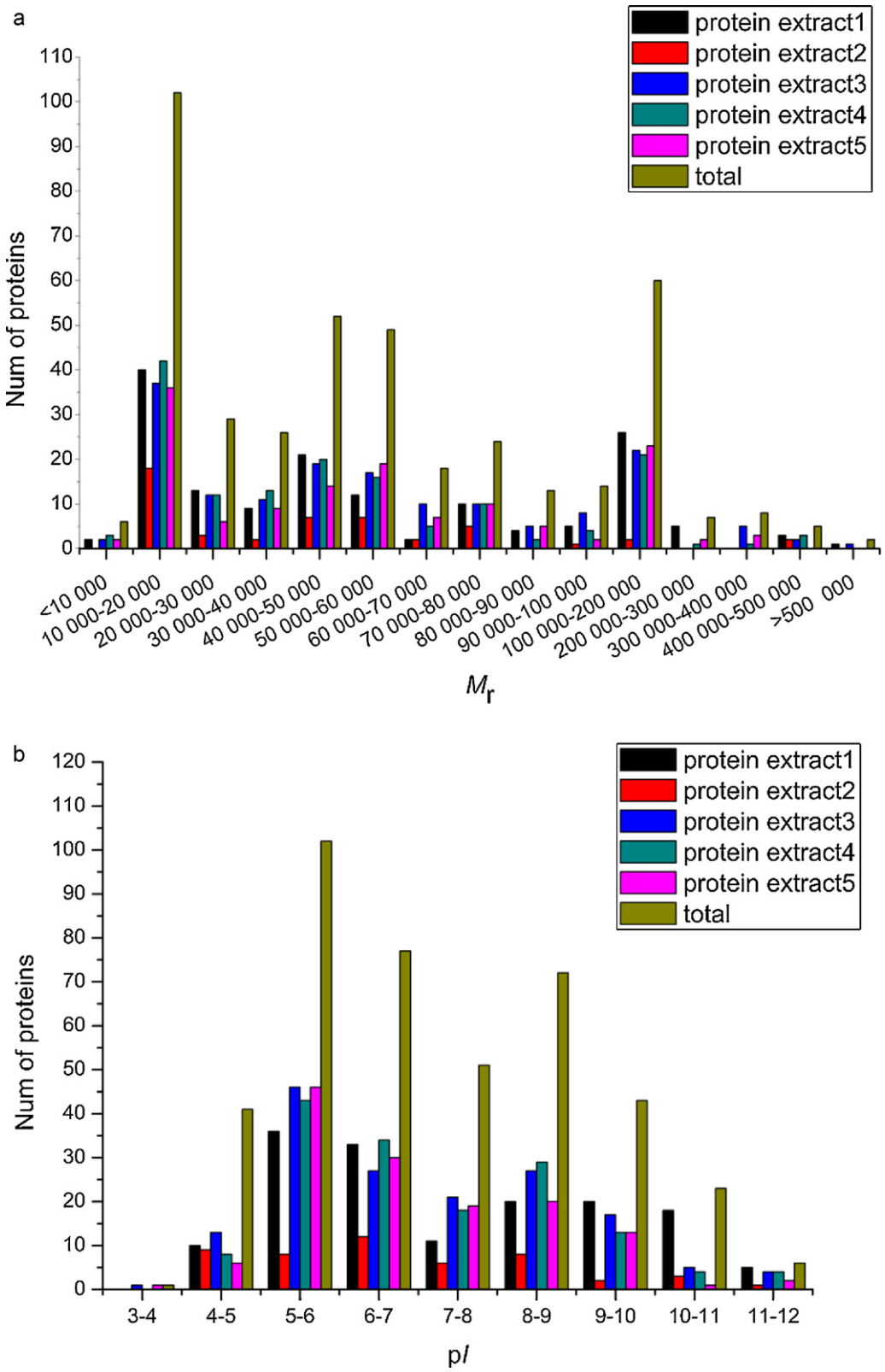

Fig. 1. M_r (a) and p*I* (b) distribution of identified proteins (□ Extract 1; Extract 2; Extract 3; Extract 4; Extract 5; Extract 6).

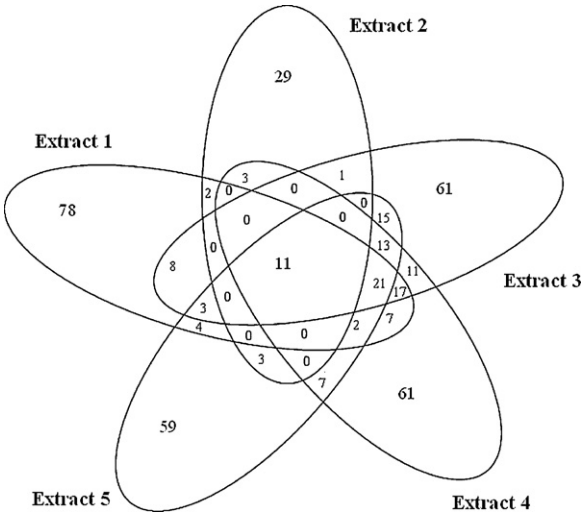

Fig. 2. Overlap of the identified proteins among the five protein extracts.

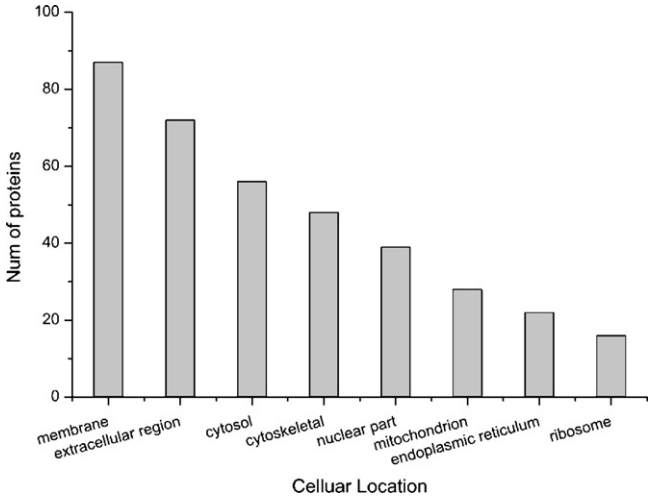

Fig. 3. Subcellular location of the proteins identified from antler of red deer (*Cervus elaphus*).

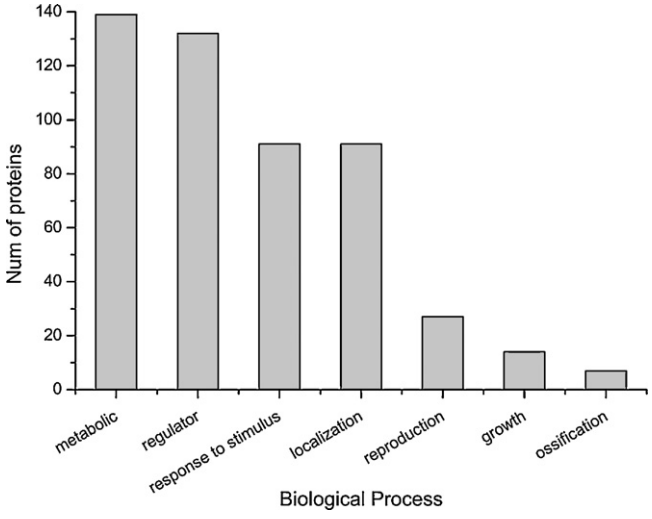

Fig. 4. Involved biological processes distribution of the proteins identified from antler of red deer (*Cervus elaphus*).

4. Conclusions

In this study, a parallel protein extraction strategy, with the application of five different kinds of lysis solutions, was developed and combined with μRPLC–ESI-MS/MS with a serially coupled long microcolumn for proteome profiling of deer antler. In total, 416 proteins were identified after searching public database with mammalian taxonomy restriction. The location and involved biological process of identified proteins were further studied. All these results demonstrate that with advanced analysis techniques, more proteome information of deer antler could be obtained. Based on this strategy, further study of antler proteome research in different growing stages was undergoing in our group, which might be of great significance for studying the developmental procedure of antler and regeneration.

Conflicts of interest

The authors have declared no conflict of interest.

Acknowledgements

The authors are grateful for the financial support from National Basic Research Program of China (2007CB914100), National Natural Science Foundation (20935004 and 20075094) and Knowledge Innovation Program of Chinese Academy of Sciences (KJCX2YW.H09).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2010.10.022.

References

[1] R.J. Goss, Nature 220 (1968) 83.
[2] R.J. Goss, Deer Antlers: Regeneration, Function and Evolution, Academic Press, New York, 1983.
[3] C. Gray, M. Hukkanen, Y.T. Konttinen, G. Terenghi, Neuroscience 50 (1992) 953.
[4] C.N. Faucheux, S.A. Horton, J.S. Price, J. Exp. Biol. 204 (2001) 443.
[5] C. Li, J.M. Suttie, Anat. Embryol. (Berl.) 204 (2001) 375.
[6] G.A. Bubenik, E. Reyes, D. Schams, A. Lobos, L. Bartos, F. Koerner, J. Exp. Zool. 292 (2002) 393.
[7] C. Li, R.P. Littlejohn, I.D. Corson, J.M. Suttie, Gen. Comp. Endocrinol. 131 (2003) 21.
[8] S.M. Francis, J.M. Suttie, J. Exp. Zool. 281 (1998) 36.
[9] S.P. Allen, M. Maden, J.S. Price, Dev. Biol. 251 (2002) 409.
[10] G.K. Barrell, R. Davies, C.I. Bailey, Reprod. Fertil. Dev. 11 (1999) 189.
[11] H.J. Park, D.H. Lee, S.G. Park, S.C. Lee, S. Cho, H.K. Kim, J.J. Kim, H. Bae, B.C. Park, Proteomics 4 (2004) 3642.
[12] Y. Fan, J. Liu, S. Wang, H. Wang, F. Shi, L. Xiong, W. He, X. Peng, Life Sci. 76 (2005) 2893.
[13] R. Pastorelli, D. Carpi, L. Airoldi, C. Chiabrando, R. Bagnati, R. Fanelli, S. Moverare, C. Ohlsson, Proteomics 5 (2005) 4936.
[14] X. Jiang, M. Ye, X. Jiang, G. Liu, S. Feng, L. Cui, H. Zou, J. Proteome Res. 6 (2007) 2287.
[15] P.H. O'Farrell, J. Biol. Chem. 250 (1975) 4007.
[16] M. Fedorova, N. Kuleva, R. Hoffmann, J. Proteome Res. 9 (2010) 2516.
[17] J. Hou, Z. Cui, Z. Xie, P. Xue, P. Wu, X. Chen, J. Li, T. Cai, F. Yang, J. Proteome Res. 9 (2010) 777.
[18] M.P. Washburn, D. Wolters, J.R. Yates, Nat. Biotechnol. 19 (2001) 242.
[19] D. Tao, G. Zhu, L. Sun, J. Ma, Z. Liang, L. Zhang, W. Zhang, Y. Zhang, Proteomics 9 (2009) 2029.
[20] F. Lestremau, A. Cooper, R. Szucs, F. David, P. Sandra, J. Chromatogr. A 1109 (2006) 191.
[21] D. Kang, H. Nam, Y. Kim, M.H. Moon, J. Chromatogr. A 1188 (2008) 208.
[22] M.M. Bradford, Anal. Biochem. 72 (1976) 248.
[23] J.D. Termine, A.B. Belcourt, P.J. Christner, K.M. Conn, M.U. Nylen, J. Biol. Chem. 255 (1980) 9760.
[24] B.R. Zeeberg, W. Feng, G. Wang, M.D. Wang, A.T. Fojo, M. Sunshine, S. Narasimhan, D.W. Kane, W.C. Reinhold, S. Lababidi, K.J. Bussey, J. Riss, J.C. Barrett, J.N. Weinstein, Genome Biol. 4 (2003) R28.
[25] M. Wendel, Y. Sommarin, D. Heinegaard, J. Cell Biol. 141 (1998) 839.
[26] A.M. Craig, J.H. Smith, D.T. Denhardt, J. Biol. Chem. 264 (1989) 9682.
[27] U.M. Wewer, R. Albrechtsen, L.W. Fisher, M.F. Young, J.D. Termine, Am. J. Pathol. 132 (1988) 345.