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An activity-maintaining sequential protein extraction method for bioactive assay and proteome analysis of velvet antlers

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ABSTRACT

The exceptional growth rate of velvet antler makes it a valuable model for studying the development of tissues, such as blood vessels, cartilage and bone. Meanwhile, investigating the activities of extracted proteins from velvet antlers promisingly leads to the discovery of new active factors which regulate the development of above-mentioned tissue types. In this study, a novel sequential protein extraction method was developed for proteome profiling and bioactivity study of velvet antlers. Herein, four antler growing tips were pooled to create a proportional pooled sample, and three aliquots of which were extracted in parallel using the developed extraction method. For each sample, proteins were extracted sequentially by saline solvent (0.15 M sodium chloride, pH 7.0), mild acid buffer (0.15 M acetate buffer, pH 4.0) and mild alkaline buffer (0.15 M glycine-sodium hydroxide buffer, pH 10.0) with good biocompatibility to prevent proteins denaturation. Then STD lysis buffer, containing 4% SDS, 0.1 M Tris–HCl and 0.1 M DTT, was used to extract hydrophobic proteins. The tryptic digest of each fraction was analyzed by nanoRPLC-ESI-MS/MS in triplicates, with false discovery rate for peptide identification adjusted to 1% to create filtered protein group list. In total, 1423 protein groups were identified, which expanded up to 3 times of the previous published dataset. The relative standard deviation of identified peptide and protein group number for all analyses indicated the good reproducibility of the developed sequential protein extraction method. Additionally, proteins extracted by acid buffer and alkaline buffer showed obvious promoting effect on the proliferation of human umbilical vein endothelial cells. All these results demonstrate that the developed sequential extraction method is efficient for the comprehensive proteome analysis and activity investigation of velvet antlers.

1. Introduction

Deer antlers are the unique organs that display an annual cycle of full regeneration in mammals [1]. In addition, antler growth is a very rapid process, with the maximum rate of elongation recorded for wapiti (*Cervus elaphus canadensis*) antlers being 2.75 cm per day [2]. During this time, the constitutive tissues, such as cartilage, bone, nerves, skin, and blood vessels also grow at the same rate [3]. Therefore, antlers are considered as valuable models for studying the signaling pathways which modulates the development of these tissues. Recent evidence suggests that antler regeneration is a stem cell-based process, and some growth factors, such as VEGF, EGF, FGF and NGF, have been proved to be involved in the exceptional growth [4–7], in which some undiscovered modulating factors with low abundance or short-half-life in development of normal tissues may be over-expressed and more likely be found. Thus, investigation on the activities of proteins extracted from velvet antlers might lead to the founding of new active factors which play roles in the development of tissues mentioned above.

To thoroughly understand the molecular mechanisms involved in the accelerated growth and to find the potential signaling molecules, it is necessary to characterize the proteome profiling of velvet antlers. Until now, only a few papers have been published on the proteome analysis of velvet antlers. Classical single-solvent was used to extract antler proteins for proteomic analysis based on 2DE and MALDI-TOF MS [8]. Total 136 proteins were identified due to insufficient protein extraction and dysfunction of 2DE on analysis of certain proteins (low abundance, acidic, basic, hydrophobic, very large, or very small). Since shotgun proteome approach is high-throughput and can avoid the intrinsic limitations of 2DE-MS analysis, in our previous work [9], a parallel-solvents protein extraction method with five different

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lysis buffers was developed, and 416 unique proteins were identified. Certainly, compared to classical single-solvent extraction methods, parallel-solvents can obtain more proteins. However, such method is sample-consuming and laboring, and results in limited protein identifications, due to inability to reduce the sample complexity.

Extensive protein extraction from tissues is one of the most critical issues for proteomic research. Nevertheless, classical single-solvent or parallel-solvents protein extraction methods are insufficient for proteome profiling and bioactivity study of velvet antlers due to the complexity of sample. In previous reports on proteome analysis of cartilage [10] and bone [11], the sequential protein extraction method improved significantly the number of identified proteins, suggesting that more proteins might be extracted if multiple complementary extraction buffers were sequentially used. In addition, many studies have demonstrated that the extracts of velvet antlers possessed widely biological activities [12–14]. For the simultaneous assessment of the bioactivities of extracted proteins, the biocompatible extraction buffers should be selected to prevent extracted proteins from denaturation. Thus, the proteomic dataset of each fraction would be used for the discoveries of active factors presented in corresponding fraction whose activities were proved by activity assay.

In the present study, a sequential protein extraction method with four complementary solvents was developed to improve the protein extraction efficiency from velvet antlers and identified the largest number of protein groups reported in velvet antler till date. In addition, proteins extracted by bio-compatible solvents showed obvious effect on proliferation of human umbilical vein endothelial cells (HUVEC). All these results demonstrated that the developed sequential protein extraction method is efficient to explore proteome profiling of velvet antlers and biological activities of extracted proteins, beneficial to find new active molecules and gain insight into the mechanisms of antler growth.

2. Materials and methods

2.1. Reagents and materials

Trypsin, urea (99.5%), Tris, ACN and formic acid (FA) were ordered from Sigma-Aldrich (St. Louis, MO, USA). BSA, DTT, IAA, and protease inhibitors cocktail were ordered from Merck (Darmstadt, GER). SDS was purchased from Biomol (Hamburg, GER). Bicinchonic acid (BCA) protein assay kit was produced by Beyotime (Haimen, Jiangsu, CN). Syringe filters and Ultra filters (10 kDa COMW) were ordered from Millipore (Bedford, MA, USA).

2.2. Sample preparation

Four antlers were harvested from 3-year-old male sika deers (*Cervus nippon Temminck*), a month after the antler regenerated from the pedicles. These antlers were cleaned with 75% ethanol (v/v) carefully, removed by a surgical hand-saw, and then stored at −80 °C after blood removal with a vacuum pump. The antler growing tips were collected according to the sampling technique described by Li et al. [15]. Briefly, the incision was made surround the antler shaft approximately 1.3 cm proximal to the antler tip to collect the tip section, which is composed of skin, reserve mesenchyme and precartilage tissues. Then, an intradermal incision was made using a scalpel on the edge of the cut surface and the incision was expanded by two pairs of fine forceps to peel off the enveloping skin.

The schematic diagram of the sequential protein extraction method was shown in Fig. 1. First, four antler growing tips were dissected into slices, washed with PBS to remove blood, and then ground into powder in a porcelain mortar in liquid nitrogen,

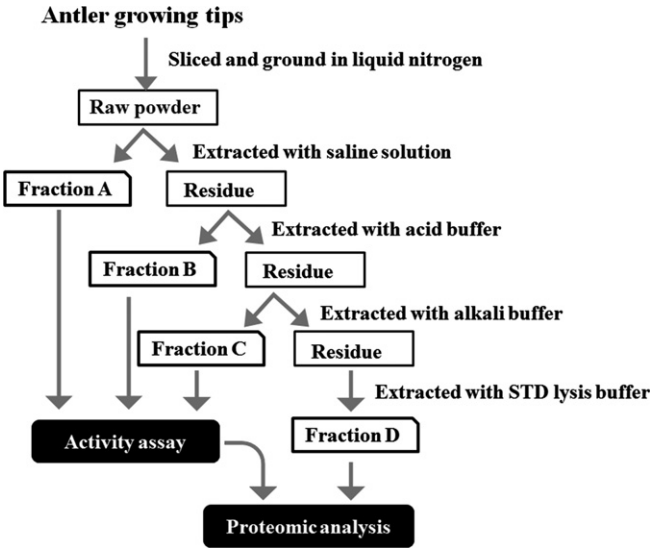

Fig. 1. Schematic diagram of sequential protein extraction method.

respectively. Equal aliquots of raw powder from four antlers were pooled to create a proportional pooled sample. Three aliquots of pooled sample were extracted in parallel using developed extraction method and named as sample 1, 2 and 3. 1 g of proportional pooled sample and 4 mL of saline solvent (0.15 M sodium chloride, pH 7.0) were put into tissue grinder for homogenization. Then the homogenate was transferred to a centrifuge tube and incubated for 30 min in an ice bath. After centrifuged at 15,000 × *g* for 20 min, the supernatant of homogenate was collected. The above process was repeated twice, and all supernatants were mixed as Fraction A. Second, the residue was extracted using mild acid buffer (0.15 M acetate buffer, pH 4.0), to obtain Fraction B. Third, the residue was extracted with mild alkaline buffer (0.15 M glycine–sodium hydroxide buffer, pH 10.0), to obtain Fraction C. In addition, the first three buffers contained 1% protease inhibitors. Finally, the residue was extracted with STD lysis buffer (4% SDS, 0.1 M Tris–HCl and 0.1 M DTT), to obtain Fraction D. Fraction A, B and C were filtrated by syringe filters and desalted using ultrafilters (10 kDa cut off) at 3000 × *g* and 4 °C, then followed by lyophilization for further activity assay and proteomic analysis. The antler proteins obtained by three aqueous buffers were digested according to our recent work [16], and Fraction D was processed according to FASP method [17] for proteomic analysis. BCA Protein Assay was used to measure the protein concentrations of extracted fractions according to reference [18].

2.3. NanoRPLC-ESI-MS/MS analysis

The tryptic digest of each fraction obtained from each sample were analyzed in triplicates by a nanoRPLC-ESI-MS/MS system, consisting of a quaternary surveyor MS pump (Thermo Fisher, San Jose, CA, USA) and an LTQ mass spectrometer (Thermo Fisher). Two micrograms of each protein digest was loaded onto a home packed capillary column (75 μm i.d. × 19 cm) in triplicate runs. Mobile phase A (0.1% FA in H_2O) and B (0.1% FA in ACN) were used to establish the 150 min gradient, comprised of 10 min of 0% B, followed by 125 min of 10–35% B, and finally maintained at 80% B for 15 min, with the flow rate at 160 nL/min. The mass spectrometer instrument was operated in positive mode with a 2.1 kV applied spray voltage. The temperature of the ion transfer capillary was set at 200 °C. One microscan was set for each MS and MS/MS scan. All MS and MS/MS spectra were acquired in the data dependent mode. A full scan MS acquired from *m*/*z* 300 to

2000 was followed by six data dependent MS/MS events. The dynamic exclusion function was set as follows: repeat count, 1; repeat duration, 30 s; exclusion duration, 60 s. The normalized collision energy for MS/MS scanning was 35%.

2.4. Cell proliferation

HUVEC cells were cultured in DMEM containing 10% FBS (v/v), and were maintained in a humidified, 95% air, and 5% CO_2 atmosphere at 37 °C. Cell proliferation was measured by MTT assay [19]. The cells were cultured in 96-well plates (2×10^3 cells/well) in different medium with samples of various concentrations (50–300 μg/mL) with deer plasma (300 μg/mL) as a reference control and PBS as blank control. The absorbance at 490 nm was recorded with the absorbance at 630 nm as reference. The optical density of each well was quantified as a percentage based on the blank control, and the data were expressed as the mean of five parallel experiments.

2.5. Data analysis

Each fraction was analyzed in triplicates by LC–MS/MS. RAW data files generated from LC–MS/MS analyses were converted to Mascot generic format (mgf) and subsequently searched using the Mascot search engine version 2.4.0. International Protein Index (IPI) Bovin v3.73 database (http://ebi.ac.uk/IPI) was used as target sequences. To evaluate the false discovery rate (FDR) for peptide and protein group identification, the decoy search was enabled, and then the target sequences were auto-appended with reverse sequences, which was used as the target/decoy database. The parameters used for database searching included methionine oxidation as a variable modification and cysteine carbamidomethylation as a fixed modification. Trypsin was selected as the protease, and a maximum of two missed cleavages were allowed. Search tolerances were set to ±2.0 Da and ±1.0 Da for the precursor ions and product ions, respectively. The false discovery rate (FDR) for peptide identification was adjusted to 1% by the built-in filter provided by Mascot 2.4.0. The following Mascot settings were used to create filtered protein group list: significance threshold (*P* value) was auto-generated when FDR of peptide identification was adjusted to 1%, expect cut-off (*E* value) was set identically as *P* value and require bold red was selected. Proteins with shared peptides were automatically grouped, and same set or subset proteins were not exported. GoMiner (http://discover.nci.nih.gov/gominer) was used to annotate and group the identified protein groups according to biological processes, molecular function and cellular location.

All the results of activity assay were expressed as mean ± standard deviation (S.D.). Data were analyzed by one-way analysis of variance (ANOVA) followed by Duncan's test for multiple comparisons. A difference of $p<0.05$ was regarded as being statistically significant. The calculations were performed with the statistical package for social sciences (SPSS) software package from SPSS Inc. (Chicago, IL, USA).

3. Results and discussion

3.1. Method development for efficient protein extraction

With the consideration on the diversity of protein properties, solvents with different pH, including saline solvent (pH 7), mild acid buffer (pH 4), and mild alkaline buffer (pH 10) were sequentially used to extract water-soluble proteins. Proteins presented in such biocompatible aqueous solvents should mainly be hydrophilic proteins with biological activities maintained. Furthermore, large amount of hydrophobic proteins in residue was incubated with STD lysis buffer to extract water-insoluble proteins.

As shown in Table 1, the total protein yields obtained from three parallel samples were 46.2 ± 1.67 mg protein/g tissue. The proportions of Fraction A, B, C and D accounted for 53.1 ± 2.30%, 1.1 ± 0.07%, 12.0 ± 0.54%, and 33.8 ± 1.11% ($n=3$) of total protein yield, respectively, which suggested that the developed sample extraction method was of good reproducibility.

For shotgun proteome analysis, proteins extracted by different solutions are digested and analyzed with nanoRPLC-ESI-MS/MS at peptide level, followed by database searching to identify proteins. Since the genome of sika deer (*Cervus nippon Temminck*) has not been sequenced completely, current public database of sika deer contained limited entries of protein sequences (only 619 entries in the Swiss-Prot database). Therefore, proteomic studies on deer followed a cross-species strategy, using a given protein database of other sequenced organism with close relative genome, analogous to the proteome profiling of *Pedobacter cryoconitis* [20], *L. starkeyi* [21] and *Marenzelleria* [22]. Since deer and bovine are very closely related in phylogenetics [23, 24], the gene sequences of cow show high similarity (in the range 90–99%) with deer genes in the coding regions of mRNAs [25, 26]. Similarly, some reports on gene expression in deer antler also revealed that the deer genes all showed high sequence homology (above 90%) to that of bovine [5, 27]. Therefore, the IPI Bovin database (v3.7.3, 30,403 entries) was selected in this study to perform a cross-species proteomic study of deer.

To increase the confidence level of identification events, the FDR for peptide identification was adjusted to 1% and that for protein group was supplied in supplementary Table 1. To assess the reproducibility of identification events, the relative standard deviations (RSDs) of identified peptide and protein group numbers for triplicate runs were calculated. As shown in supplementary Tables 2 and 3, the overwhelming majority of RSDs of peptide and protein group identification for triplicate runs were less than 7.9%, except for that of fraction A from sample 1 (the RSDs of peptide and protein group identification were 11.84% and 8.47%, respectively). Furthermore, the reproducibility of peptide and

Table 1
Quantity of extracted proteins and identified number of peptides and protein groups obtained from different extraction fractions and samples.

Items	Fraction A ($n=3$)	Fraction B ($n=3$)	Fraction C ($n=3$)	Fraction D ($n=3$)	Samples ($n=3$)
	(mean ± SD RSD)	(mean ± SD RSD)	(mean ± SD RSD)	(mean ± SD RSD)	(mean ± SD RSD)
Quantity of protein (mg/g tissue)	24.53 ± 1.06 4.32%	0.49 ± 0.03 6.12%	5.55 ± 0.25 4.50%	15.63 ± 0.51 3.26%	46.20 ± 1.67 3.61%
Number of identified peptides	715 ± 33 4.62%	389 ± 41 10.54%	1388 ± 117 8.43%	1803 ± 156 8.65%	2879 ± 199 6.91%
Number of identified protein groups	329 ± 14 4.26%	163 ± 17 10.43%	437 ± 8 1.83%	614 ± 30 4.89%	985 ± 30 3.05%

protein group identification events among triplicate fractions and samples were also calculated (shown in Table 1). Except for fraction B, other fractions and samples showed the RSDs less than 8.7%. These results demonstrated the good reproducibility of the combination of developed sample preparation method and nanoRPLC-ESI-MS/MS analysis.

After the database searching results of the four sequential fractions from sample 1 were combined, 999 proteins were identified, among which 118 (11.8%), 68 (6.8%), 110 (11.0%), and 329 (32.9%) proteins were uniquely identified in Fractions A, B, C and D (shown in Fig. 2A), and only 41 (4.1%) were found in all four fractions, indicating that these extraction steps were complementary and more comprehensive proteomic profiling of anlter growing tip might be obtained with sequential protein extraction method. Furthermore, the technical reproducibility of developed method was assessed by parallel analysis of triplicate samples, as shown in Fig. 2B and C. The similar overlaps of identified proteins from each analysis could be obtained, showing the good reproducibility among three parallel extraction processes.

With the combination of data searching results of three parallel samples, 1423 protein groups were identified with at least one unique peptide from the antler growing tip (Supplementary Tables 4 and 5). This is the largest protein dataset till date obtained from velvet antlers, which should be contributed to high efficiency of the developed sequential protein extraction strategy. To further improve the reliability of protein identification, protein groups matched with at least two peptides were summarized in Supplementary Table 4, and 669 protein groups were identified, based on which gene ontology analysis was performed with high confidence.

3.2. Characterization of proteins identified from velvet antlers

The distribution of relative molecular masses (M_r) and isoelectric points (*pI*) of identified proteins were classified. The *pI*s of identified proteins ranged from 3.98 to 11.87, and the M_r ranged from 5.6 to 77.7 kDa (Supplementary Fig. 1). Not surprisingly, in the identified protein list, some structural proteins (e.g., vimentin, actin and tubulin) and abundant mammalian cell proteins (e.g., myosin and dynein) were included. In addition, several serum-derived proteins (e.g., serum albumin, complement, and fibrinogen) were undesirably identified for their tightly binding to the hydroxyapatite crystals. Apart from these, the identified proteins exhibited a broad spectrum of functions. To further characterize these proteins, GoMiner program was used to perform the annotation, which has been widely used to predict the locations and functions of proteins [28].

3.2.1. Cellular location

The cellular location of 466 proteins was identified (Supplementary Fig. 2A), which distributed over almost every cell compartment. Notably, 213 proteins were located in membrane, which could mediate many vital cellular processes [29]. The identification of such a large number of membrane-related proteins which were ignored in previous study attributed to STD lysis buffer, which was efficient to extract hydrophobic proteins. Furthermore, the intracellular location of identified proteins was shown in Supplementary Fig. 2B. The primary location was cytosol, with 136 proteins (29.2%) identified. Besides, the number of proteins located in mitochondrion (102), cytoskeleton (88) and endoplasmic reticulum (74) were also substantial, followed by location at nucleolus, ribosome and golgi. The result provided comprehensive information of proteins with various subcellular locations, indicating that protein extraction performed with these buffers was extensive.

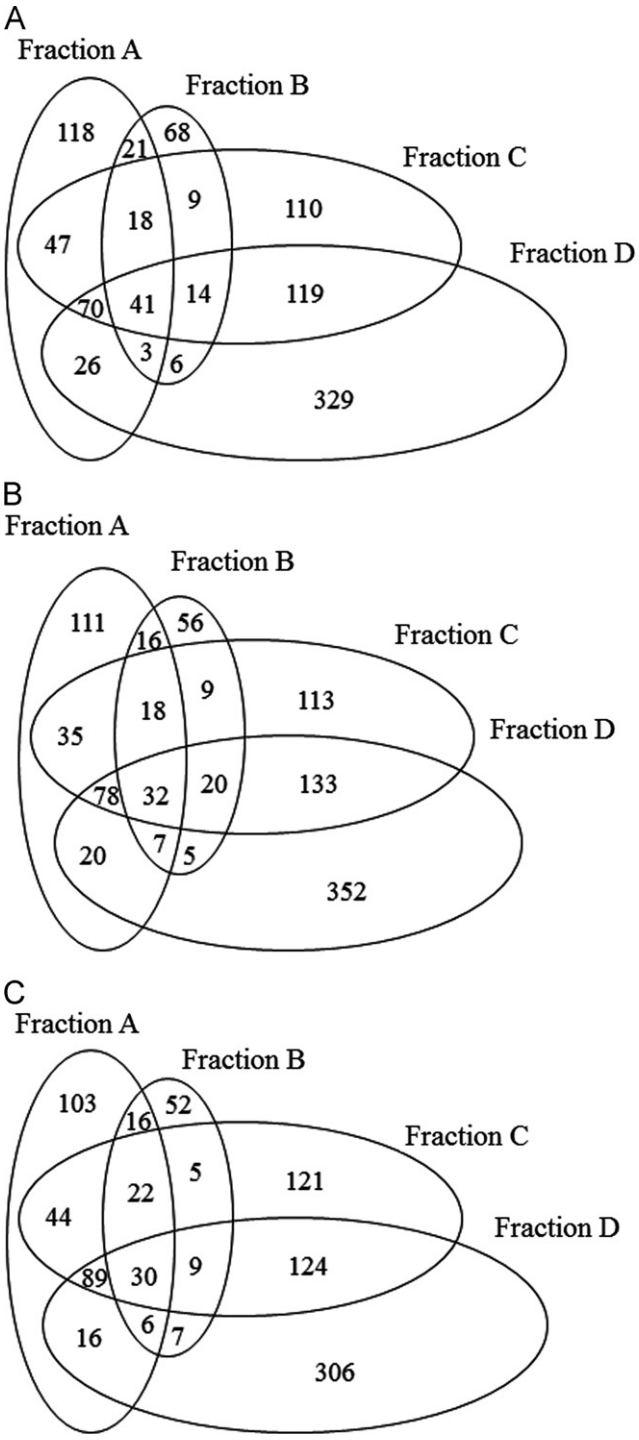

Fig. 2. Venn-diagram showing overlap of proteins identified by sequential protein extraction strategy for Samples 1 (A), 2 (B) and 3 (C).

3.2.2. Molecular function

Totally, 472 identified proteins were annotated in molecular function, as presented in Supplementary Fig. 3. The majority (93.9%) were grouped in binding function, which is vital in interactions between the various systems of cell, and is also believed to be auxiliary to perform protein biological functions.

Besides, 228 proteins related to enzymatic activity were identified, among which, 124 proteins belonged to the class of hydrolases activity, including 30 peptidase activity-related proteins (Supplementary Table 6). In addition, 12 protease inhibitors, such as serpin H1, serpin A3-1 and triosephosphate isomerase,

were also identified, which are indispensable in the regulation of protein metabolism, and could prevent unwanted proteolysis. Furthermore, 106 structural proteins were identified. This group contained 49 structural constituents of ribosome (Supplementary Table 7), which is the workhorse of protein biosynthesis. So many structural proteins of ribosome were first identified in velvet antlers, possibly indicating the strong presence of ribosomes and vigorous production of proteins.

Moreover, some proteins associated with protein biosynthesis were also identified, including 37 transcription regulator proteins and 21 translational regulator molecules (Supplementary Table 8). The identification of above-mentioned proteins, as well as their putative functions, is consistent with the physiological phenomenon of the robust development of velvet antlers. In addition, many proteins involved in energy generation were found in the identified proteins list, including 18 electron transport chain (ETC) related proteins and 8 adenosine triphosphate (ATP) synthase related proteins (Supplementary Table 9). ETC is the cellular mechanisms used for extracting energy from redox reactions, such as the oxidation of sugars (respiration). ATP synthase converts mechanical into chemical energy by producing ATP, which powers most cellular reactions. A large number of identified proteins involved in energy regeneration confirm that sufficient energy support is essential to promote the rapid growth of velvet antlers.

3.2.3. Biological process

As shown in supplementary Fig. 4, 462 identified proteins were annotated in biological process, which could be classified into several biological processes. The largest category (320 proteins) was metabolism, which contained proteins involved in biosynthetic and catabolic processes of protein, carbohydrate, lipid and hormone. Among them, proteins involved in protein biosynthesis and catabolism was in majority and contained many of the above-mentioned enzymes. In the second largest category, 250 proteins associated with biological regulation were observed. This category could be categorized into three types: regulation of biological process (229), regulation of biological quality (92) and regulation of biological function (36 proteins). In addition, a large group of 176 proteins was assigned a role in development process. Among them, 10 blood vessel morphogenesis proteins, 9 cartilage development proteins, 7 bone development proteins and 20 neuron development proteins were identified (Supplementary Table 10). The identification of these kinds of tissue-specific proteins is consistent with the histological characteristics of velvet antlers, which is an organ and mainly composed of skin, blood vessel, cartilage and bone tissue.

Interestingly, 66 identified proteins were grouped into "death", among which 59 apoptosis related proteins were annotated (Supplementary Table 11). This might be due to the programmed cell death (apoptosis) in antler growing tip, where apoptosis presents a higher percentage in antler growing tip than that recorded in any other adult tissue [30]. Besides proteins above-mentioned, 190, 156 and 117 proteins involved in the biological processes of localization, response to stimulus and cellular component biogenesis were identified, respectively.

3.3. Effects of sequential fractions from antler growing tips on cell proliferation

The bioactivities of fractions obtained by saline solvent, mild acid buffer and mild alkaline buffer were examined, with the result of PBS treated group as basal value. Since the STD lysis buffer could denature proteins, the corresponding fractions were excluded from activity assay.

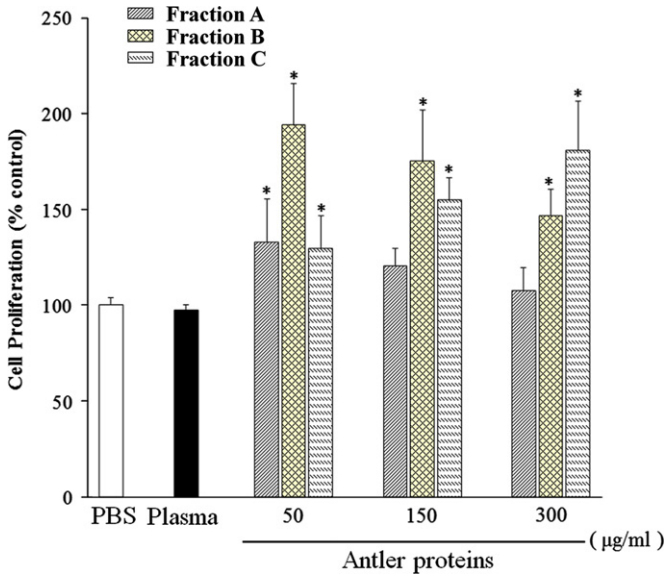

Fig. 3. Effects of fractions extracted from antler growing tips on proliferation of HUVEC cells. The results are shown as percentage of the control ± standard deviation (S.D.) (*$p < 0.05$ vs. control).

As shown in Fig. 3, Fraction A, B and C all showed proliferation promoting activities. Fraction C showed activity in concentration-dependent manner and increased proliferation rate to 180.9% of the basal value at 300 μg/mL concentration. However, Fraction A and B worked in a reverse concentration-dependent manner. In those cases, Fraction A and B at 50 μg/mL presented the highest activity, and increased proliferation rate to 133.0% and 193.4% of the basal value, respectively. We thought that these fractions might contain some components with proliferation-promoting activity as well as some proliferation inhibitors. At the lower concentration (50 μg/mL) in growth medium, content of inhibitors is too low for its effect manifestation, and the evident proliferation of the cell culture is observed. At the higher concentration (300 μg/mL), the content of inhibitors reaches the "working" value, and this partly neutralizes the effect of promoters, leading to the slowing down of cell proliferation. The proliferation rates of deer plasma treated groups are similar with that of PBS treated groups, suggesting that the real active factors present in fractions but not deer plasma. The high proliferation-promoting activities of fractions derived from antler growing tip manifested that these extraction buffers were biocompatible and could maintain protein activities. Meanwhile, the fact that different fractions work in different manners further confirmed the efficient fractionation capacity of the sequential extraction method.

These results are consistent with the reports that precartilage region in antler tip is characterized by impressively proliferation of the vascular associated cells [4], and is likely to contain factors that specifically regulate the growth of blood vessels. Additionally, pleiotrophin [5], an angiogenic factor, was found in this study, which might contribute to the promoting effect on proliferation of HUVEC.

4. Conclusion

A sequential protein extraction method was developed for the proteome analysis of velvet antlers, followed by analysis with nanoRPLC-ESI-MS/MS, by which the protein identification efficiency was improved significantly. Three aliquots of pooled antler growing tip samples were extracted in parallel and the tryptic

digest of each fraction was analyzed in triplicates. The RSD of identified peptide and protein group number demonstrated the good reproducibility of developed protein extraction method. The low overlap of the identified proteins of different fractions indicated these sequential extraction buffers had good complementarity, which could not only reduce the complexity of each fraction, but also contribute to the complete extraction of protein. Meanwhile, the former three extraction buffers are biocompatible and the corresponding fractions show different promoting effect on proliferation of HUVEC cells. The method developed in this work enabled investigation on biological activity and proteome profiling of velvet antler simultaneously, which can help us to find new active factors produce effect on antler growth and gain insight into the mechanisms of antler growth.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.01.015.

References

[1] C.Y. Li, Birth Defects Res. C 96 (2012) 51–62.
[2] R.J. Goss, Clin. Orthop. Relat. R. 69 (1970) 227–238.
[3] D.E. Clark, C. Li, W. Wang, S.K. Martin, J.M. Suttie, Anat. Rec. Part A 288A (2006) 973–981.
[4] D.E. Clark, E.A. Lord, J.M. Suttie, Anat. Rec. Part A 288A (2006) 1281–1293.
[5] P.M. Barling, A.K.W. Lai, L.F.B. Nicholson, Cell Biol. Int. 29 (2005) 229–236.
[6] A.K.W. Lai, W.L. Hou, D.J. Verdon, L.F.B. Nicholson, P.M. Barling, Tissue Cell 39 (2007) 35–46.
[7] W. Pita-Thomas, M. Nieto-Sampedro, R.M. Maza, M. Nieto-Diaz, J. Neurosci. Res. 88 (2010) 3034–3047.
[8] H.J. Park, D.H. Lee, S.G. Park, S.C. Lee, S. Cho, H.K. Kim, J.J. Kim, H. Bael, B.C. Park, Proteomics 4 (2004) 3642–3653.
[9] L. Gao, D.Y. Tao, Y.C. Shan, Z. Liang, L.H. Zhang, Y.S. Hou, J. Chromatogr. B 878 (2010) 3370–3374.
[10] R. Wilson, A.F. Diseberg, L. Gordon, S. Zivkovic, L. Tatarczuch, E.J. Mackie, J.J. Gorman, J.F. Bateman, Mol. Cell. Proteomics 9 (2010) 1296–1313.
[11] X.G. Jiang, M.L. Ye, X.N. Jiang, G.P. Liu, S. Feng, L. Cui, H.F. Zou, J. Proteome Res. 6 (2007) 2287–2294.
[12] H.S. Lee, M.K. Kim, Y.K. Kim, E.Y. Jung, C.S. Park, M.J. Woo, S.H. Lee, J.S. Kim, H.J. Suh, J. Ethnopharmocol. 133 (2011) 710–717.
[13] S.S. Roh, M.H. Lee, Y.L. Hwang, H.H. Song, M.H. Jin, S.G. Park, C.K. Lee, C.D. Kim, T.J. Yoon, Ann. Dermatol. 22 (2010) 173–179.
[14] Z.H. Yang, L.J. Gu, D.L. Zhang, Z. Li, J.J. Li, M.R. Lee, C.Y. Wang, Z. Wang, J.H. Cho, C.K. Cung, Asian Aus. J. Anim. 25 (2012) 708–716.
[15] C.Y. Li, D.E. Clark, E.A. Lord, J.A.L. Stanton, J.M. Suttie, Anat. Rec. 268 (2002) 125–130.
[16] J.R. Wisniewski, A. Zougman, N. Nagaraj, M. Mann, Nat. Methods 6 (2009) 359–360.
[17] L.L. Sun, D.Y. Tao, B. Han, J.F. Ma, G.J. Zhu, Z. Liang, Y.C. Shan, L.H. Zhang, Y.K. Zhang, Anal. Bioanal. Chem. 399 (2011) 3387–3397.
[18] E.V. Piletska, S.S. Piletsky, M.J. Whitcombe, I. Chianella, S.A. Piletsky, Anal. Chem. 84 (2012) 2038–2043.
[19] Y. Cetin, L.B. Bullerman, J. Agr. Food Chem. 53 (2005) 6558–6563.
[20] A.G. Pereira-Medrano, R. Margesin, P.C. Wright, Proteomics 12 (2012) 775–789.
[21] H.W. Liu, X. Zhao, F.J. Wang, X.N. Jiang, S.F. Zhang, M.L. Ye, Z.B.K. Zhao, H.F. Zou, Fems Yeast Res. 11 (2011) 42–51.
[22] M. Blank, S. Mikkat, M. Verleih, R. Bastrop, J. Proteome Res. 11 (2012) 897–905.
[23] K. Wada, M. Nishibori, M. Yokohama, Small Ruminant Res. 69 (2007) 46–54.
[24] D.S. Gallagher, J.N. Derr, J.E. Womack, J. Hered. 85 (1994) 204–210.
[25] V. Steger, A. Molnar, A. Borsy, I. Gyurjan, Z. Szabolcsi, G. Dancs, J. Molnar, P. Papp, J. Nagy, L. Puskas, E. Barta, Z. Zomborszky, P. Horn, J. Podani, S. Semsey, P. Lakatos, L. Orosz, Mol. Genet. Genomics 284 (2010) 273–287.
[26] I.J. Gyurjan, A. Molnar, A. Borsy, V. Steger, L.J. Hackler, Z. Zomborszky, P. Papp, E. Duda, F. Deak, P. Lakatos, L.G. Puskas, L. Orosz, Mol. Genet. Genomics 277 (2007) 221–235.
[27] Y.P. Yin, L.N. Tang, J.B. Zhang, B. Tang, Z.Y. Li, Plos One 6 (2011) e20929.
[28] S. Briesemeister, T. Blum, S. Brady, Y. Lam, O. Kohlbacher, H. Shatkay, J. Proteome Res. 8 (2009) 5363–5366.
[29] E. Wallin, G. von Heijne, Protein Sci. 7 (1998) 1029–1038.
[30] M. Colitti, S.P. Allen, J.S. Price, J. Anat. 207 (2005) 339–351.