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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 bio-compatibility 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.

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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, 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].

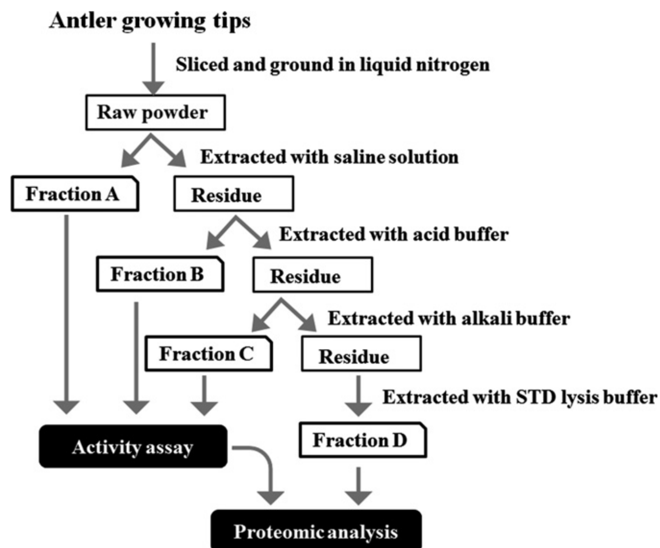

Fig. 1. Schematic diagram of sequential protein extraction method.

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

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