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Ionic liquid-based method for direct proteome characterization of velvet antler cartilage

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ABSTRACT

The cartilage zone of the velvet antler is richly vascularized, this being a major difference to the classical cartilage, in which there are no blood vessels. Angiogenesis and rapid growth of vasculature in velvet antler cartilage (VAC) make it an ideal model for discovering the novel angiogenic regulatory factors. However, the proteomic analysis of VAC is challenging due to the serious interference of proteoglycans (PGs) and collagens. To achieve a comprehensive proteome characterization of VAC, herein, we developed an ionic liquid-based method using 1-dodecyl-3-methylimidazolium chloride ([C12-mim]Cl) for selective extraction of cellular proteins from VAC. Compared with the previous cetylpyridinium chloride (CPC)-based method, the developed [C12-mim]Cl-based method takes much less processing time, shows facile preparation procedure and good compatibility towards downstream proteomic analysis, leading to the identification of more protein groups (1543 vs 753), membrane proteins (663 vs 279) and transmembrane proteins (217 vs 58).

1. Introduction

Deer antlers, as complex mammalian appendages, are the unique organs that display an annual cycle of full regeneration in mammals [1]. Histology shows that the velvet antler is composed of several tissues, such as velvet-like skin, cartilage, bone, nerves and blood vessels [2]. The antler of deer grows at a remarkable rate of 1–2 cm per day, with the maximum record of 2.75 cm in wapiti (*Cervus elaphus*) [3]. In this process, the tissues mentioned above could also grow at the same rate. Thus, velvet antler needs a well-developed blood vessel network to support the rapid growth. Angiography also reveals that the arterial vessels of the antler originate from branches of the superficial temporal artery while the venous vessels return in parallel arrays through the cartilaginous core of the antler [4]. Therefore, the rapid growth of blood vessels make velvet antler cartilage (VAC) a valuable model for screening novel angiogenic regulators and exploring mechanisms modulating the growth of vasculature.

Unlike the classical cartilage, the VAC is highly vascularized. As seen in either transverse or sagittal section (Fig. 1), the VAC possesses numerous blood vessels, which are separated from each other by the chondrocyte columns. To maintain their structure, the chondrocytes produce a large amount of cartilaginous matrix, which consists mainly of proteoglycans (PGs) and collagens. However, these high-abundance components can heavily mask the cellular proteins, especially the low-abundance membrane proteins, leading to serious interference in subsequent proteome characterization [5]. To exclude these high-abundance components, cetylpyridinium chloride (CPC) was used to selectively precipitate PGs from extracting solution of human articular cartilage [6]. However, the residual CPC could decrease the efficiency of trypsin digestion and lead to ion suppression during mass spectrometry analysis. Therefore, methanol/chloroform precipitation was performed to remove CPC, which might inevitably result in the loss of some proteinous components, such as proteolipids and soluble proteins [7,8]. Therefore, the comprehensive proteome characterization of cartilage necessitates more effective protein extraction method.

In our previous studies, 1-dodecyl-3-methylimidazolium chloride ([C12-mim]Cl) has been successfully applied to membrane proteomic analysis and shows an excellent capacity in dissolving hydrophobic proteins [9–11]. In this work, we proposed a facile method for selective extraction of proteins from VAC. Firstly,

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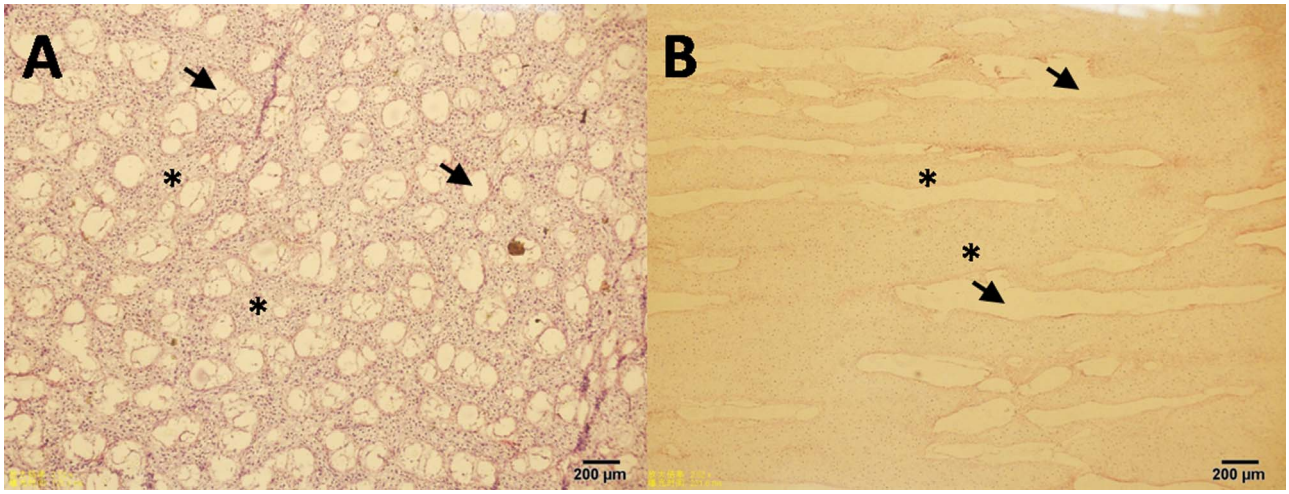

Fig. 1. Numerous blood vessels (arrow) and chondrocyte columns (asterisk) in the velvet antler cartilage. H+E staining, scale bar=200 μm. A: Transverse section. B: Sagittal section.

VAC was cut into 10-μm slices and directly extracted by 4% (m/v) [C12-mim]Cl solution. After a short-time agitation and ultrasonication, the samples were processed using filter-aided sample preparation (FASP) method [12], and finally analyzed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). Compared with the previous CPC-based method, the [C12-mim]Cl-based method exhibits a dramatic improvement in the identification of peptides (6530 vs 2968), protein groups (1543 vs 753), membrane proteins (663 vs 279) and transmembrane proteins (217 vs 58) from VAC, indicating the great potential of the [C12-mim]Cl-based method in protein extraction from cartilaginous tissues, as well as good compatibility and availability for subsequent proteomic analysis.

2. Materials and methods

2.1. Reagents and materials

Trypsin (bovine pancreas), urea, CPC, ammonium bicarbonate (ABC) and formic acid (FA) were ordered from Sigma-Aldrich (St. Louis, MO). Dithiothreitol (DTT) and iodoacetamide (IAA) were from Acros (Morris Plains, NJ). Protease inhibitor cocktail and acetonitrile (ACN, HPLC grade) were ordered from Merck (Darmstadt, Germany). [C12-mim]Cl was obtained from Shanghai Chengjie Chemical Co. Ltd. (Shanghai, China). BCA assay kit was purchased from Beyotime Biotechnology (Shanghai, China).

Ultrafilters (10 kDa) were from Sartorius Stedim Biotech (Goettingen, Germany). Fused-silica capillaries (75 μm i.d. × 365 μm o.d.) were bought from Sino Sumtech (Handan, China). Hypersil C18 silica particles (5 μm, 300 Å pore) were obtained from Thermo (San Jose, CA). Deionized water purified by a Milli-Q system from Millipore (Bedford, MA) was used in all experiments. All other chemicals and solvents were analytical grade.

2.2. Preparation of protein samples

Velvet antlers were harvested from three male sika deer (*Cervus nippon*), almost 30 days after the antler regenerated from the pedicles in May (late spring in the northern hemisphere). The velvet antlers were cleaned with 75% ethanol (v/v) carefully, removed by cutting the proximal region with a surgical hand-saw and dissected into distinct zones according to the previous report [13]. After the removal of blood, the cartilage zones were washed with phosphate-buffered saline (PBS, 1% protease inhibitor cocktail, pH 7.2), frozen on a HM440E cryotome (Kalamazoo, MI) at −30 °C and cut into 10-μm slices. Cartilage slices from 3 deer were pooled together and identical aliquots (200 mg) were extracted separately by [C12-mim]Cl-based method and CPC-based method, respectively. Each method was performed in three biological replicates.

For [C12-mim]Cl-based method, tissue slices were suspended in 4% [C12-mim]Cl (m/v, 1% (v/v) protease inhibitor cocktail) at 100 mg cartilage/mL and agitated for 5 min. Then, slices were discarded and the extracting solutions were ultrasonicated for 120 s (10 s intervals every 5 s). After centrifugation (20,000 g for 5 min), the supernatant was collected and the BCA assay was employed for protein concentration measurement. For CPC-based method, the experimental procedure used was based on the previous report by Vincourt et al. [6]. In brief, tissue slices were suspended in extraction buffer (500 mM NaCl, 50 mM HEPES, 1% (v/v) protease inhibitor cocktail, pH 7.2) at 100 mg cartilage/mL and agitated for 1 h. The extracting solution was clarified (6,000 g for 5 min) and then CPC was added at a final concentration of 1% (m/v) to aggregate with PGs for 1 h. After centrifugation at 6,000 g for 5 min, the supernatant was collected and subjected to methanol/chloroform precipitation [7]. The protein pellets were washed and resuspended in methanol and dried depending further treatment. All experiments were performed at room temperature.

The protein samples obtained from the two methods were processed using the FASP method with minor modifications [12]. All centrifugation steps were performed at 14,000 g and 20 °C unless otherwise specified. In brief, proteins were reduced and denatured by adding DTT and boiling at 95 °C for 3 min. Then, about 100 μg proteins were transferred to a 10 kDa centrifugal spin filter and sequentially washed with 200 μL of 8 M urea for 3 times to remove the ionic liquids ([C12-mim]Cl or CPC). Afterwards, samples were alkylated with IAA for 40 min in darkness at room temperature and washed with 200 μL of 20 mM ABC for two times. Next, tryptic digestion was performed by adding trypsin (Promega) at 1:30 (enzyme/substrate, m/m) in 200 μL of 20 mM ABC and incubating at 37 °C for 14 h. Peptides were recovered by transferring the filter to a new collection tube and spinning. Filter was rinsed twice with 100 μL of 20 mM ABC to complete peptide recovery, and the completed filtrate was desalted by a C18 precolumn. Finally, the collected peptides were dried with a SpeedVac and redissolved in 0.1% (v/v) FA aqueous solution for further nanoRPLC-MS/MS analysis.