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Chondroitin sulphate extracted from antler cartilage using high hydrostatic pressure and enzymatic hydrolysis

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ABSTRACT

Chondroitin sulphate (CS), a major glycosaminoglycan, is an essential component of the extracellular matrix in cartilaginous tissues. Wapiti velvet antlers are a rich source of these molecules. The purpose of the present study was to develop an effective isolation procedure of CS from fresh velvet antlers using a combination of high hydrostatic pressure (100 MPa) and enzymatic hydrolysis (papain). High CS extractability (95.1 ± 2.5%) of total uronic acid was obtained following incubation (4 h at 50 °C) with papain at pH 6.0 in 100 MPa compared to low extractability (19 ± 1.1%) in ambient pressure (0.1 MPa). Antler CS fractions were isolated by Sephacryl S-300 chromatography and identified by western blot using an anti-CS monoclonal antibody. The antler CS fraction did not aggregate with hyaluronic acid in CL-2B chromatography and possessed DPPH radical scavenging activity at 78.3 ± 1.5%. The results indicated that high hydrostatic pressure and enzymatic hydrolysis procedure may be a useful tool for the isolation of CS from antler cartilaginous tissues.

1. Introduction

Antlers from deer species have unique mammalian structures, where there is annual occurrence of cycle of growth, maturation, mineralisation, casting and regeneration [4]. Growing antlers are composed of different types of tissues including cartilaginous and osseous tissues surrounded by velvet connective tissues. Cartilage in antlers comprises of collagen and proteoglycans as its major extracellular matrix constituents, and aggrecan as the predominant proteoglycan [27,12]. The structure of aggrecan has a protein core of approximately 200 kDa molecular weight in which glycosaminoglycan (GAG) chains containing approximately, 100 chondroitin sulphate (CS) chains (MW 10–25 kDa), 30–60 keratin sulphate (KS) chains (MW 3–15 kDa), and N- and O-linked oligosaccharides are covalently attached [10]. CS is one of the GAGs composed of the alternating sugars D-glucuronic acid (GlcA) and *N*-acetyl-D-galactosamine (GalNAc). As a major GAG of aggrecan molecules in the antler, CS accounts for approximately 92% of total GAGs with relatively small amounts of KS [29,25]. Thus, CS is an important component of the extracellular matrix in antler cartilage.

Due to its negative charge, CS is responsible for water retention in the cartilage, which is important for pressure resistance. Physiologically, CS increases hyaluronan production by human synovial cells to maintain viscosity in the synovial fluid [6]. It also has many functional properties for the prevention of osteoarthritis, such as modifying the chondrocyte apoptosis process, improving the anabolic/catabolic balance of the extracellular cartilage matrix, reducing pro-inflammatory and catabolic factors, and stimulating the anabolic processes involved in new cartilage formation in osteoarthritis [11]. In addition, CS shows a dose-dependent increase in free radical scavenging [2]. This antioxidant activity, caused by the chelation of transition metals such as Cu^{2+} and Fe^{2+}, is also believed to be partially responsible for the chondroprotective effects of CS, as oxidative stress has been shown to increase the risk and effects of osteoarthritis [1,7,3,5,33]. CS is an important constituent for the preservation of corneal tissues. So far, there is no efficient treatment that could prevent the pathological process of arthropathy. Oral administration of CS was suggested to be beneficial in the treatment of osteoarthritis. To take advantage of these important functionalities, CS can be ingested as a food supplement once it has been extracted from the cartilaginous tissue. The extraction of CS requires the degradation of collagen and the core protein in the extracellular matrix.

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In this study, a combination of high hydrostatic pressure (HHP) and enzymatic hydrolysis (HHP-EH) is tested as a relatively new extraction process for isolating CS from cartilaginous tissues of antlers. HHP greater than 100 MPa increases water penetration into the protein interior and damages the cell membrane, which unfolds protein molecules and simultaneously inactivates bacteria at ambient temperatures within few minutes. This phenomenon allows HHP to be widely used in food preservation as an alternative to heat treatment, maintaining the stability and functionality of enzymes at a pressure less than 200 MPa and concurrently increasing their reaction rate [17]. For example, the catalytic activity of thermolysin increased 45-fold at 200 MPa [14] and that of α-chymotrypsin increased 7-fold at 150 MPa [19] as compared to ambient pressure.

Antler cartilaginous tissues are of relative low-value but are abundantly available in nonedible by-products rich in CS-proteoglycan, collagen and glycoprotein. There are few reports about the isolation of CS from antler cartilage and its antioxidant ability. The aim of this work was to determine the effect of high pressure, temperature and incubation time on the catalytic activity of papain for extracting CS as a potential antioxidant agent. GAGs can be directly extracted from tissues by hydrolysis with exogenous enzymes like papain or pronase [20]. Further separation of the CS in the crude extract was obtained by column chromatography, and the hyaluronic acid binding ability of CS was also examined.

2. Materials and methods

2.1. Preparation of antlers

Samples of antlers were obtained from 4-year-old wapiti stags at a local elk farm (Leduc, Alberta, Canada). The main beam of each harvested antler was skinned and divided into 4 sections (tip, upper, middle and base) as previously described [28]. Macroscopically, the tip section contains pre-chondroblast soft cartilaginous tissue with no bony structure. Most of the upper section comprises cartilaginous chondrocytes with minor osteoblasts. In contrast, bones are the major tissues found in the middle and base sections. Only the tip and upper sections were selected and transported to the laboratory on ice rinsed with cold water, dissected free of non-cartilaginous adherent connective tissues, and stored at −20 °C until extracted. Five hundred grams of frozen samples were then thawed at 4 °C, chopped into small pieces, added to 500 mL of deionised water, homogenised with a blender (Waring commercial, MX1500XTS model, Stamford, CT, USA) and then sieved through a 100-mesh screen. The unscreened particles were further liquefied using a colloid mill (Chemineer Inc., W200V model, Dayton Ohio, USA). The suspensions from blending and milling were combined and stored for further use.

2.2. High hydrostatic pressure and enzymatic hydrolysis (HHP-EH) treatment

HHP-EH treatments were performed in a portable scale high hydrostatic pressure system (TFS-2 L, Toyo-Koatsu Innoway Co. Ltd., Hiroshima, Japan) with a cylindrical pressure chamber, which has a volumetric capacity of 2 L. 500 mL of the suspension (modified at pH 6.0) was then mixed with papain type 111 (4 mg/g of tissue, EC3.4.22.2, Sigma–Aldrich, USA). First, the liquid mixtures of antler samples and enzyme were poured into 5 plastic ziplock bags (10 mL per bag) and sealed. Deionised water was used as the pressurisation medium in the HHP unit. Test samples were subjected to HHP treatment at selected pressures of 0.1, 25, 50, 75 and 100 MPa for 4 h at 50 °C. Secondly, five bags were subjected to HHP treatment for different incubation times of 1, 2, 3, 4, and 8 h at 50 °C at 100 MPa. Four bags were also subjected to HHP treatment for different temperatures of 20, 30, 40 and 50 °C for 4 h at 100 MPa. Following HHP treatment, the pressure was quickly released and the extracts were boiled for 10 min to inactivate the papain. After cooling, the cooled extract was centrifuged (5000 × *g* for 10 min) and then filtered through a Whatman no.5 filter paper. The extract was stored at −20 °C until analysed. The residual tissue was further digested with papain, and uronic acid contents in both the extract and the residual tissue were determined by the carbazole reaction (see Section 2.7). These estimates enabled the proportion of uronic acid liberated to be expressed as a percentage of the total uronic acid recovered. The total extractability of uronic acid was then compared between the different extraction conditions. The preparation of each extract, which was referred to as antler papain extract, was performed in triplicate and the entire experiment was independently replicated three times to address precision.

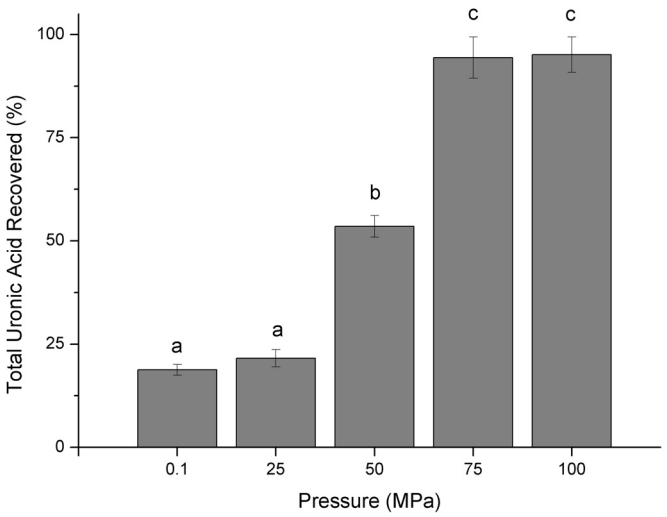

Fig. 1. Effect of pressure on the content of uronic acid by using high hydrostatic pressure and enzymatic hydrolysis at 50 °C for 4 h. Values are means of triplicate. Error bars shown are standard errors of the means ($n = 3$). Letters indicate significant differences among pressure treatments.

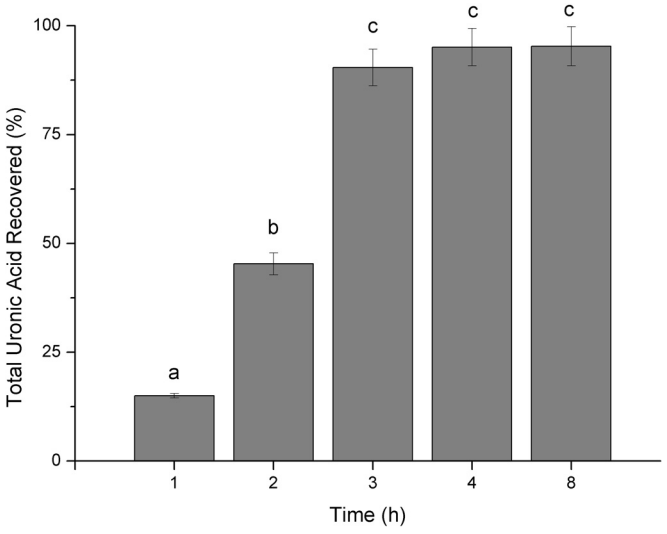

Fig. 2. Effect of incubation time on the content of uronic acid by using high hydrostatic pressure and enzymatic hydrolysis at 50 °C under 100 MPa. Values are means of triplicate. Error bars shown are standard errors of the means ($n = 3$). Letters indicate significant differences among incubation times.