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Sulfated glycosaminoglycan-derived oligosaccharides produced from chicken connective tissue promote iron uptake in a human intestinal Caco-2 cell line

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ABSTRACT

Food grade sulfated glycosaminoglycan (GAG) polysaccharides were successfully extracted from chicken cartilage and skin. Their respective oligosaccharides were obtained by bovine testicular hyaluronidase digestion. The antioxidant capacity was analyzed to determine the possible mechanism explaining how GAGs promote iron uptake by the Caco-2 cells. The sulfated GAG oligosaccharides derived from cartilage possessed the greatest DPPH scavenging and ferric reducing activities ($p < 0.05$), but limited ferrous chelating activities. Both the cartilage and skin sulfated GAG polysaccharides showed greater ferritin formation compared to the negative control ($p < 0.05$). Depolymerisation of GAG polysaccharides to oligosaccharides further improved ferritin formation by twofold. This research establishes that chicken sulfated GAG polysaccharides can enhance iron uptake by Caco-2 cells. The enhanced iron uptake through enzymatic GAG depolymerisation could be due to the combined effects of reduced molecular weight, increased amount of hydroxyl terminal groups and ferric reducing activities.

1. Introduction

The broiler chicken meat processing industry produces a large amount of by-products rich in connective tissue (i.e. keel cartilage, skin, and bone residues), which can be exploited for isolating important natural health ingredients (Nakano, Betti, & Pietrasik, 2010). Connective tissue contains the extracellular matrix that includes collagens, glycoproteins, proteoglycans and glycosaminoglycans (GAGs). Proteoglycans are major components of this extracellular matrix and are comprised of two types of molecules: the glycosylated protein core and covalently attached sulfated GAGs. These acid polysaccharides from various animal sources have been extensively studied. They are described based on their disaccharide composition and degree of sulfation; on one hand, the sulfated GAGs, such as chondroitin sulfate (CS), dermatan sulfate (DS), keratan sulfate (KS) and heparan sulfate (HS) are all bound glycans to the protein core; and on the other hand, hyaluronic acid (HA), the non-sulfated GAG polysaccharides, all exist as a free polymer (Nakano et al., 2010).

Sulfated GAG polysaccharides can be liberated from the extracellular matrix by enzymatic or chemical hydrolysis and are considered biologically active compounds. They have a wide range of applications in pharmaceutical and food industries. For instance, CS is used as a supplement for treating osteoarthritis due to its anti-inflammatory and chondro-protective effects (Dean et al., 1991). CS has also been used as an emulsifying agent in mayonnaise (Hamano, Mitsuhashi, Acki, & Yamamoto, 1989). HS and DS also have anticoagulant activities (Volpi, 2006).

Besides these known properties, recent *in vitro* studies suggest that GAG polysaccharides can enhance non-heme iron absorption, thereby improving one's nutritional iron status (Huh, Hotchkiss, Brouillette, & Glahn, 2004; Laparra, Barbera, Alegria, Glahn, & Miller, 2009; Laparra, Tako, Glahn, & Miller, 2008). For instance, the GAG-containing fraction of cooked haddock has increased the iron uptake of epithelial Caco-2 cells in a simulated gastrointestinal model (Huh et al., 2004; Laparra et al., 2008). However, an *in vivo* study using commercial HA and CS did not improve inorganic iron absorption in young women (Storcksdieck, Walczyk, Renggli, & Hurrell, 2007). Jin and Glahn (2007) pointed out that GAGs isolated from foods have different structures and lower molecular weights (<5 kDa) than the commercially purified GAGs (HA at MW of 1000–1200 kDa and CS at MW of 20 kDa, respectively). This could be why contradicting results have been obtained so far.

Glahn, Lee, Yeung, Goldman, and Miller (1998) demonstrated that ferritin synthesis in Caco-2 cells combined with the *in vitro*

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gastrointestinal digestion is a good model for studying the iron bioavailability. However, the mechanisms regarding sulfated GAGs-mediated Fe uptake by Caco-2 cells are still ambiguous. It is well known that non-heme iron availability increases in the presence of natural antioxidants, like for instance ascorbic acid (vitamin C). This molecule has a 2, 3-enediol structure which can reduce the ferric (Fe^{3+}) ions into the more soluble ferrous (Fe^{2+}) form that can be subsequently readily internalized into the epithelial cells via the divalent metal transporter (DMT-1) system (Jin et al., 2009). Polysaccharides, in general, have some reducing power, which can be enhanced with chemical modification such as sulfation. Sulfated polysaccharides have greater antioxidant and reducing powers because the sulfate groups facilitate a weaker dissociation energy of the hydrogen bonds between the polysaccharide chains. This then promotes the donation of hydrogens from the hydroxyl groups (Wang, Hu, Nie, Yu, & Xie, 2015). Besides this particular reducing capacity, Laparra et al. (2009) also suggested that Fe could be internalized into cells by endocytosis due to the formation of Fe^{3+} – GAG polysaccharide complexes. This is likely due to the carboxylic acid and sulfated moieties, which cause GAGs to act as chelating agents that improve iron uptake by intestinal epithelial cells (Huh et al., 2004; Laparra et al., 2008). However, GAG polysaccharides with a large molecular mass and charge density have raised concerns about their possible poor intestinal absorption, which would impair their therapeutic utility (Baici et al., 1992). Recent studies have demonstrated that reducing the CS molecular weight could be a possible way to improve the intestinal absorption of the polysaccharides (Qian et al., 2013; Xiao et al., 2014). Hence depolymerisation of GAGs to produce low molecular weight oligosaccharides may be beneficial to promote their absorption and ultimately increase the up-take of iron through a GAG-oligosaccharide mediated inorganic iron complexes by Caco-2 cells. Furthermore, it is expected that creating these low molecular weight oligosaccharides through depolymerisation would generate more hydroxyl terminal groups. These groups would then have a greater capacity to increase both the radical scavenging capacity and the ability to reduce Fe^{3+} to Fe^{2+} with a subsequent positive effect on iron availability (Wang et al., 2015).

To the best our knowledge, there have been no studies demonstrating the effect of enzymatic depolymerisation of sulfated GAG polysaccharides from avian sources on iron uptake by Caco-2 cells and how this relates to their antioxidant capacity. The objectives of this study were: (1) to characterize purified sulfated GAGs from broiler chicken cartilage and skin obtained through a food grade extraction system; (2) to compare the antioxidant capacity of extracted sulfated GAGs before and after enzymatic depolymerisation; (3) to determine the effect of enzymatic depolymerisation of the sulfated GAGs on Fe uptake by Caco-2 cells.

2. Materials and methods

2.1. Materials

Ten whole broiler chicken carcasses were obtained from a local food store (Edmonton, AB, Canada). Cartilage samples were collected from the anterior and posterior sternum, distal femur, proximal tibia and proximal humerus (Nakano, Pietrasik, Ozimek, & Betti, 2012). Skin samples were collected from whole carcasses. Chicken skin and cartilage were cut into small pieces and pretreated separately. All samples were lyophilized and stored at 4 °C until analyzed. Pancreatin from porcine pancreas, bovine testicular hyaluronidase (EC3.2.1.35), chondroitinase-ABC (EC4.2.2.4) from *Proteus vulgaris*, chondroitinase-AC (EC4.2.2.5) from *Flavobacterium heparinum*, porcine pepsin (EC3.4.4.1), bile extract, hyaluronic acid from rooster comb and chondroitin sulfate B (DS) from porcine intestinal mucosa were obtained from Sigma–Aldrich (Mississauga, ON, Canada). Chelex-100 was from Bio-Rad Laboratories (Hercules, CA, USA). Standard GAGs, including chondroitin sulfate A (CS) from bovine trachea (H1913), were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Diethylaminoethyl (DEAE) cellulose anion exchanger (DEAE Sephacel) was obtained from GE Healthcare, Bio-Science (Mississauga, ON, Canada). Cellulose acetate strips (CA-MEMM) for electrophoresis were obtained from Topac Inc. (Cohasset, MA, USA).

All chemicals were of analytical grade, and solutions were prepared with Milli-Q purified water (17 MΩ·cm, Millipore, Bedford, MA, USA).

2.2. Experimental design

Sulfated GAGs were liberated from broiler chicken skin and cartilage connective tissues by pancreatic proteolysis and then ultrafiltration for removing the soluble peptides with a 10 kDa molecular weight. The digests were precipitated with 70% ethanol. The total GAGs precipitate were dissolved in deionized water and were then fractionated by using anion exchange chromatography with sequential elution with 0.4 M and 2.0 M NaCl. Three independent extractions of sulfated GAG polysaccharides were conducted from chicken skin and cartilage. Part of the extracted GAGs were then digested using testicular hyaluronidase. Hence a total of 4 treatments (intact and digested GAGs from cartilage + intact and digested GAGs from skin) were obtained resulting in a total of 12 samples (3 replications × 4 treatments). Along with these treaments, commercial laboratory grade CS from bovine trachea and DS from porcine intestinal mucosa were also treated similarly and used as standard references. Subsequently, DPPH scavenging activities, ferric reducing activities and iron chelating activities were determined to estimate the antioxidant capacities of the intact and depolymerized GAGs. In these tests, ascorbic acid and EDTA were used as positive controls. In the second part of the study, the effect of test samples on Fe uptake by the Caco-2 cell culture model after simulated gastrointestinal digestion was investigated. Test samples were the same as previously described. A solution of $FeCl_3$ was used as a negative control while a solution $FeCl_3$ was used as a positive control.

2.3. Food-grade extraction and separation of sulfated GAG polysaccharides

As previously reported, food-grade methods have been used to extract and fractionate sulfated GAGs from broiler chicken biomass (Nakano et al., 2012).

Chicken skins were washed with distilled water (1:5, w/v) and stirred for 20 min to remove excess blood and some impurities. Then they were soaked in a 0.1 M NaOH solution at a ratio of 1:10 (w/v) for 6 h by changing the NaOH solution every 2 h to remove myofibrillar muscle proteins. The alkaline treated skin was rinsed well with water, and then defatted by stirring with 90% ethanol at a ratio of 1:10 (w/v) for 24 h by changing the ethanol every 6 h. The cartilage was washed with distilled water to remove blood and adhering muscle tissues. The chicken skin and cartilage were digested with pancreatic enzymes. Tissue samples were first boiled in water (1:5, w/v), cooled to room temperature and then homogenized. Pancreatin was added to the homogenate at 1% sample weight, and the pH was adjusted to 7.6. The mixture was incubated at 50 °C overnight. After proteolysis, the mixture was boiled for 5 min for enzyme inactivation and then cooled to room temperature. Fat was removed by centrifugation using an Avanti J-E centrifuge (Beckman Coulter Inc., Palo Alto, CA, USA) at 11,300×*g* at 24 °C for 20 min followed by filtration through Whatman No.1 filter paper. The soluble digest was passed through