Bioproduction of sulfated glycosaminoglycan oligosaccharides and their effect on non-heme iron uptake: Studies in human intestinal cell lines (Caco-2 cells)

by

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Abstract

Glycosaminoglycans (GAGs) such as chondroitin sulfate (CS), dermatan sulfate (DS), heparin (HP), heparan sulfate (HS), and non-sulfated hyaluronic acid (HA) are a group of complex polysaccharides exhibiting a wide range of biological functions. These macromolecules are structurally heterogeneous depending on the animal source, and their functional properties may also be affected by the specific purification procedure. The overall thesis objective is to investigate the bioproduction of GAG-derived sulfated oligosaccharides and study their effect on iron uptake by Caco-2 cells. The first study aimed to develop a food-grade approach for the extraction of GAGs from chicken cartilage and skin, which are low-value poultry by-products. Purified sulfated GAGs were successfully extracted from chicken cartilage (CS) and skin (CS/DS) using porcine pancreatin (a food-grade proteolysis) followed by ethanol precipitation and purification with anion exchange chromatography. In the second stage of this study, the antioxidant capacity of these chicken sulfated GAG polysaccharides and their respective oligosaccharides (oligos) obtained by enzymatic digestion were evaluated. Bovine trachea CS and porcine intestinal DS were used as controls. Then, their effect on iron uptake was evaluated by ferritin formation using an *in vitro* digestion in Caco-2 cell model. The CS-oligos derived from both CS polysaccharides of chicken and bovine sources possessed the greatest DPPH scavenging and ferric reducing activities ($p < 0.05$) but had limited ferrous chelating activities. Both chicken sulfated GAG polysaccharides and their controls showed greater ferritin formation compared to the blank ($p < 0.05$). Depolymerisation of CS polysaccharides further improved ferritin formation by two-fold in both the sample and control. The enhanced iron uptake through enzymatic CS depolymerisation may be due to the combined effects of reduced molecular weight, increased amount of hydroxyl terminal groups, and ferric reducing activities. Based on these results, the second study focused on

supplementing enzymatic depolymerised bovine trachea CS into skim bovine milk (SBM) to verify if its promoting effect on Fe uptake can remain pronounced. The effect of CS-oligos with various SBM fractions on Fe uptake by an *in vitro* digestion/Caco-2 cell model was evaluated. SBM was separated into casein, whey, lactose and oligosaccharides by ultrafiltration. Modified fractions representing the absence of either casein, whey, or lactose were then prepared. Extrinsic ferrous sulfate was added since the intrinsic iron content of SBM is relatively low. The effect of milk oligosaccharides on Fe uptake was evaluated for the first time, which was similar to that of whey. The ferritin synthesis rate was 1.5-fold greater in SBM after the addition of CS-oligos. Its enhancing effect was the most prominent with milk oligosaccharides and the least with casein. The results suggest the possibility of supplementing CS-oligos as a dietary intervention strategy to address iron deficiency anemia. However, the costly enzymes would limit the possibility of implementing such a technology for commercial opportunities. The last study evaluated possible chemical depolymerisation methods. Bovine trachea CS was hydrolyzed under acid, alkaline and combined (acid and alkaline) conditions at 60°C. The combined acid- and alkaline-treated CS possessed the greatest antioxidant capacity. All chemical hydrolyzed CS samples showed greater ferritin formation compared to the blank ($p < 0.05$), where the greatest ferritin level was achieved by the combined treated CS. Overall, this thesis provides a research foundation for producing high quality CS and CS-oligos from the animal sources, as an individual compound or in a food mixture, that can enhance Fe uptake in a Caco-2 cell model. It demonstrates the potential of animal CS and CS-oligos to act as a supplement with multiple bioactivities.

Preface

This thesis is an original work of He Nan Wang. It is presented in manuscript format and consists of six chapters.

The introduction (Chapter 1) describes the research background, hypothesis and objectives of this work.

Chapter 2 is the literature review and focuses on the function of glycosaminoglycans, the current production methods for bioactive glycosaminoglycans and oligosaccharides, the therapeutic applications of antioxidant glycosaminoglycans and their effect on improving non-heme iron bioavailability

Chapter 3 to chapter 5 constitute the experimental studies.

Chapter 3 was published as Wang, H. and Betti, M. (2017). Sulfated glycosaminoglycan-derived oligosaccharides produced from chicken connective tissue promote iron uptake in a human intestinal Caco-2 cell line. *Food Chemistry.* 220, 460-469.

Chapter 4 was published as Wang, H. and Betti, M. (2018). Supplementation of chondroitin sulfate-oligosaccharides in skim bovine milk improves Fe uptake in a human intestinal Caco-2 cell line. *Journal of Functional Foods.* 46. 556-566.

Chapter 5 was submitted for publication as Wang, H. and Betti, M. (2018) and is entitled, "Chemical depolymerisation of chondroitin sulfate glycosaminoglycan improves both antioxidant activity and Fe uptake in a human intestinal Caco-2 cell model".

The conclusion (Chapter 6) describes the outcomes of this research, with a brief discussion on the implications of the results and suggestions for future research in this field.

I was responsible for the experimental design, data collection, analyses and manuscripts preparation in all the published and submitted manuscripts. Dr. N. Takuo assisted with the proofreading of Chapters 3 and 4. Dr. Béla Reiz assisted with the mass spectrometry analysis of chapter 4. Dr. M. Betti was the supervisory author and was directly involved with concept formation and manuscript composition.

"Indeed, the management of iron in our body has been compared to the handling of fire in our homes. Confinement of fire to the furnace, the fireplace, and the stove provides essential services. Allowing fire to invade the walls of our homes ensures destruction."

Eugene D. Weinberg

Dedication

To my parents, Manfu Wang and Yanjun Sun

Whose love has always been encouraging me to go forward

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Abbreviation

Abs – absorbance

AD- Alzheimer's disease

Aβ - amyloid-β peptide

ANOVA - analysis of variance

AA- ascorbic acid

AMAC- 2-Aminoacridone

BSA – bovine serum albumin

CNS- central nervous system

CS-chondroitin sulfate

CPC- cetylpyridinium chloride

cGMP-Current Good Manufacturing Practice

C4S-chondroitin-4-sulfate

C6S-chondroitin-6-sulfate

CPP- caseinophoesphopeptides

Caco-2 cell- continuous cell of heterogeneous human epithelial colorectal adenocarcinoma cells

DP- degree of polymerization

DPPH-2, 2-Diphenyl-1-picrylhydazyl

DcytB-Duodenal cytochrome B

DMT1- Divalent metal transporter

DS-dermatan sulfate

DEAE- Diethylaminoethyl

DMEM- Dulbecco's Modified Eagle Medium

DMSO- dimethyl-sulfoxide

DD- The degree of deacetylation

DTT-thiol- dithiothreitol

ESI-Electrospray ionisation

ECM- extracellular matrices

EDTA- ethylenediaminetetraacetic acid

EtOH-ethanol

EULAR- European League Against Rheumatism

E. coli- Escherichia coli

FLD-fluorescence detection

GAGs- glycosaminoglycans

GlcNAc- *N*-acetyl-D-glucosamine

GalNAc- *N*-acetyl-D-galactosamine

GlcA- glucuronic acid

GSH-Glutathione

HPLC – high performance liquid chromatography

HP-heparin

HS-heparan sulfate

HA- hyaluronic acid

HILIC- hydrophilic interaction

HEPES- 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

IdoA- iduronic acid

IDA- iron deficiency anaemia

KS- keratan sulfate

LC-Liquid chromatography

LMWH- Low molecular weight heparin

LMWCS- low molecular weight chondroitin sulfate

LDL- Low-density lipoprotein

NDD- neurodegenerative diseases

NTBI- non-transferrin-bond iron

^{1}H NMR-Proton nuclear magnetic resonance

MWCO- molecular weight cut off

MS – mass spectrometry

MS/MS- liquid chromatography tandem mass spectrometry

MTT-(3-[4,5-dimethylthiazol-2-yl]-2,3-diphenyl tetrazolium bromide)

MEM- minimal essential medium

MALDI-TOF- Matrix-assisted laser desorption/ionization time-of-flight

MDA- malondialdehyde

OA- osteoarthritis

OPA-o-phthalaldehyde

OARSI- Osteoarthritis Research Society International

PD- Parkinson's disease

ROS- reactive oxygen species

SD- standard deviation

SYSADOA- symptomatic slow acting drug

SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis

SBM- skim bovine milk

TEER- Trans-epithelial electrical resistance

UP-DF- ultrafiltration/diafiltration

UV- Ultraviolet

Δdi-4S-Chondroitin disaccharide 4S

Δdi-6S- Chondroitin disaccharide 6S

Δdi-0S- Chondroitin disaccharide 0S

Δ4,5-unsaturated uronic acid residue-glycosilic linkage cleavage carbon position

Chapter 1. General Introduction and Objectives

Glycosaminoglycans are a group of complex anionic polysaccharides including chondroitin sulfate (CS), dermatan sulfate, heparin (HP), heparan sulfate (HS), keratan sulfate (KS) and hyaluronic acid (HA) that are found in the extracellular matrix (ECM) of virtually all mammalian tissues. All GAGs, except HA, are distributed as sulfated glycan sidechains of proteoglycans (protein-GAG complexes). Their ability to bind and alter protein–protein interactions or enzymatic activity has led to their identification as important bioactive macromolecules (Volpi, 2006). In addition to the well known therapeutic applications of these macromolecules, such as CS being used as a supplement for treating osteoarthritis, HP/HS and DS have been demonstrated to act as anticoagulant agents. HA possesses gel-like properties and is able to provide functional support for tissues. The emergence of improved enzymatic and analytical tools for the study of this complex sugar has increased the understanding of the GAG structure-activity relationship and has led to the discovery of new functions from this old novel bioactive molecule.

Due to the increasing number of GAG applications, their commercial demands have been increasing significantly in the last decade and the global GAG (CS, HP/HS and HA) market is expected to continuously grow in the next decade (Badril, Linhardt and Koffas, 2018). The main sources for current commercial GAG production include shark and bovine cartilage as well as porcine intestines. The expanded GAG markets reveal a need to find alternative suitable and more affordable starting materials for GAG extraction. The broiler meat industry processing by-products represent a large amount of underutilized connective tissues. Components like skin, cartilage and bone residues represent an under-explored and potential source for GAG extraction. The concentration and purity of the extracted GAGs from commercial products directly influences their application and therefore market price. Different chemical compounds (i.e. cetylpyridinium

chloride and trichloroacetic acid) and detergents used for GAG isolation may not suitable for human consumption. In addition, peptides, proteins and nucleic acids extracted from the animal tissues are common contaminations of the GAG extract (Volpi, 2009). Thus, a food-grade approach for a GAG extraction method is needed to ensure a high yield, low-cost process to maintain the quality and purity of GAGs.

Moreover, the *in vitro* data suggest that the carbohydrate fraction of the cooked fish meat, in particular sulfated GAGs, may play a role to enhance dietary iron absorption, thereby improving iron nutritional status (Huh, Hotchkiss, Brouillette and Glahn, 2004; Laparra, Tako, Glahn and Miller, 2008; Laparra, Barbera, Alegria, Glahn and Miller, 2009). These studies suggest that the low molecular weight (< 5 kDa) carbohydrate fraction with the presence of CS/DS-related structures is released from the GAG polysaccharides due to the low pH of the simulated gastro-digestion and has shown a positive effect on improving the non-heme iron uptake by the intestinal epithelial (Caco-2) cells (Huh et al., 2004; Laparra et al., 2008). On the contrary, an *in vivo* study indicated that commercial HA and CS at much higher molecular weights (HA at MW of 1000–1200 kDa and CS at MW of 20 kDa, respectively) did not have an effect on non-heme iron absorption in young women (Storcksdieck, Walczyk, Renggli and Hurrell, 2007). This disparity could be explained by the structural analyses demonstrated by Huh et al (2004) and Laparra et al (2008), which suggested that the GAG polysaccharide isolated from cooked fish displayed different structures and lower molecular weights than the commercial standards. It has been speculated by these authors that the purified sulfated GAGs from fish maintain iron in a stable soluble form suitable for absorption and may even be internalized via endocytosis by the Caco-2 cells (Laparra et al., 2009). However, due to the absence of specific glycosidases and sulfatases secreted in the pancreatic juice or present on the intestinal wall (Barthe, Woodley and Lavit, 2004),

the GAG polysaccharides are not expected to be extensively digested when they reach the small intestine. The mechanisms regarding how sulfated GAGs promote iron bioavailability has remained ambiguous. Research on the effect of sulfated GAGs on iron bioavailability is also very limited to date. The question of whether the structure-activity relationship of sulfated GAGs is ultimately involved in their ability to improve iron absorption in human intestinal cells still needs to be answered.

All these considerations provide a rationale to investigate the animal-derived sulfated GAG-mediated enhancement of non-heme iron absorption by human intestinal epithelial (Caco-2) cells. Therefore, during my five-year PhD program, firstly I was dedicated to confirm the possibility of producing high quality sulfated GAG polysaccharides from underutilized poultry by-products via a novel food-grade approach and, secondly, I worked in obtaining enzymatic depolymerised oligosaccharides from GAGs. The antioxidant capacity of the sulfated GAG polysaccharides and their sequential oligosaccharides were analysed and used to determine the possible mechanism of how GAGs promote iron uptake by the Caco-2 cells. These studies aimed to prove that the poultry by-products represent a valuable source for food-grade extraction of high quality sulfated GAGs. The antioxidant activities of the sulfated GAG polysaccharides and their derivatives were related to their effect on iron uptake by the Caco-2 cell model, which may explain the structure-activity relationship that is contributing to enhancing non-heme iron absorption. After that, I was also interested in verifying if the effect of the sulfated GAGs and their derivatives could remain pronounced when applied to a food product like milk. I was eager to know what mechanisms may be present that would confer the enhancement of non-heme iron absorption, especially in food that has poor iron bioavailability. The last part of my research focused on possible chemical depolymerisation pathways to produce a novel Fe absorption-enhancing sulfate oligosaccharide

product from commercial bovine trachea CS, to possibly implement such a technology toward commercial opportunities.

The ultimate goal of this research was to evaluate whether the sulfated GAGs extracted from the animal by-products have the potential to act as a supplement with multiple bioactivities. In order to achieve this long-term objective, the studies were conducted in this PhD thesis with specific objectives are as follows:

1. Study 1 and 2 were combined and are presented in Chapter. The hypothesis is that the sulfated glycosaminoglycan-derived oligosaccharides produced from chicken connective tissue promote iron uptake in a human intestinal Caco-2 cell line. The chicken cartilage, skin and dark meat were chosen to represent the different by-product tissues. After optimizing the food-grade extraction process, high quality CS and DS were successfully extracted from chicken cartilage and skin and used for further functional property analyses.

   Specific objectives were:

   a) To study the antioxidant activities of the sulfated GAGs (CS and DS) extracted from different collagen-rich poultry tissues and their effect on Fe uptake by Caco-2 cells with *in vitro* digestion model;

   b) To evaluate the effect of enzymatic polymerisation on these GAG polysaccharide structures in relation to their antioxidant activities and their effect on Fe uptake by an *in vitro* digestion/Caco-2 cell model.

2. Study 3 is presented in Chapter 4. The hypothesis is that the supplementation of chondroitin sulfate-oligosaccharide in skim bovine milk improves Fe uptake in a human intestinal Caco-2 cell line. The commercial skim bovine milk (SBM) was chosen mainly due to its poor iron bioavailability. How naturally existing milk components can contribute (enhance or inhibit) to iron bioavailability still needs to be investigated.

Specific objectives are:

a) To evaluate the effect of individual SBM components including casein, whey, lactose and milk oligosaccharides on Fe uptake by using an *in vitro* digestion/Caco-2 cell model;

b) To determine the effect of bovine trachea CS-oligosaccharides on the iron bioavailability when supplemented with SBM, modified SBM fraction (i.e. "SBM minus lactose", "SBM minus casein" etc.) and individual SBM components using an *in vitro* digestion/Caco-2 cell model.

3. Study 4 is presented in Chapter 5. The hypothesis is that the chemical depolymerisation of chondroitin sulfate glycosaminoglycan improves both antioxidant activity and Fe uptake in a human intestinal Caco-2 cell model. Bovine trachea CS was hydrolyzed under acid, alkaline and combined (acid and alkaline) conditions at 60°C.

   Specific objectives were:

   a) To evaluate the physio-chemical properties of depolymerized CS products under both acid, alkaline and combined (acid and alkaline) treatments;

   b) To compare the antioxidant capacity of these resulting CS-hydrolysates and their effect on iron bioavailability using *in vitro* digestion/Caco-2 cell model.

Chapter 2. Literature Review

2.1. Structure and functions of Glycosaminoglycans (GAGs)

Glycosaminoglycans (GAGs) are a group of structurally related, naturally occurring polysaccharides, found as the carbohydrate moieties of proteoglycans (PG) and sometimes as free polysaccharides. They are expressed ubiquitously on animal cell surfaces and within extracellular matrices (ECM), interact with a wide range of proteins and contribute to their biomechanical and biological properties in both physiological and pathological processes (Jackson, Busch and Carin, 1991; Casu and Lindahl, 2001). The GAG chains are composed of alternating units of an amino sugar, either *N*-acetyl-D-glucosamine (GlcNAc) or *N*-acetyl-D-galactosamine (GalNAc) and a uronic acid, either glucuronic acid (GlcA) or iduronic acid (IdoA), except keratan sulfate, which has a galactose instead of the uronic acid. The amino sugar is in most cases acetylated, except in heparin, and the uronic acid can be either GlcA or its isomer IdoA (Figure 2.1).

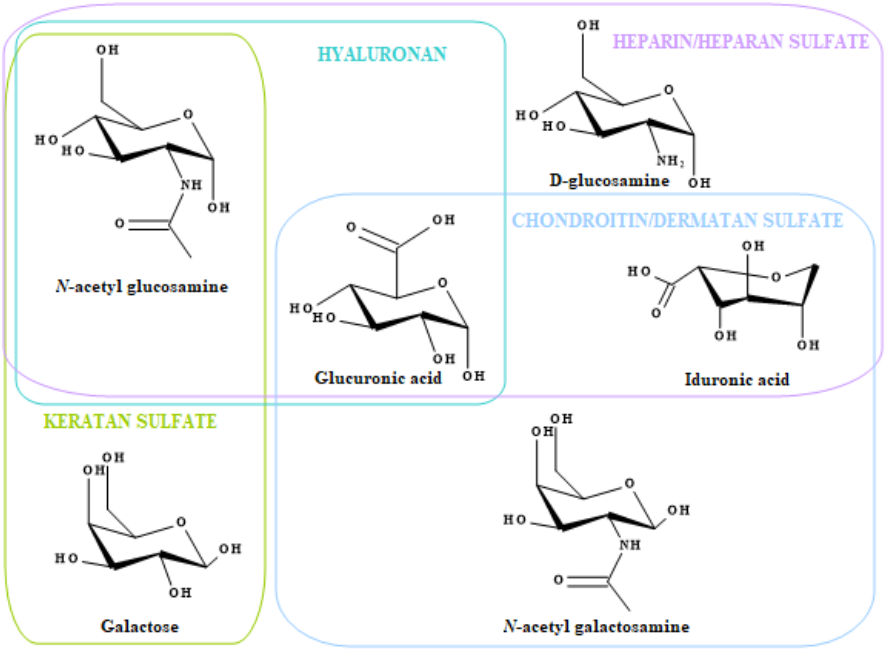

Figure 2. 1. Monosaccharides of GAGs and the different combinations of disaccharide units in different types of GAGs (use non-sulfated monosaccharides as example). The graph is from Valcarcela et al., (2017), with permission from Elsevier press.

These repeating disaccharide units of GAG polysaccharide chains are negatively charged and coupled through glycosidic linkages (α or β) into long linear/acidic chains with variable lengths and composition (amino sugar, acidic sugars, and sulfate groups). The carbon backbone of the GAG chain may undergo no further modifications (i.e. hyaluronic acid) or may be further modified through sulfation (i.e. heparin, heparan sulfate, chondroitin sulfate, dermatan sulfate and keratan sulfate). Sulfation in keratan sulfate occurs exclusively at C-6 of the GlcNAc ring, whereas other sulphated GAGs display diversity in the position of sulfate groups. The basic structure and composition of the major commercial GAGs, chondroitin sulfate (CS)/dermatan sulfate (DS) heparin (HP)/heparan sulfate (HS), keratan sulfate (KS) and hyaluronic acid (HA) are shown in

Table 2.1.Family of glycosaminoglycans. The table is reproduced from Datta and Linhardt (2017) and Xiong, Li and Hou (2012).

Type	Disaccharide unit	Chemical formula	Sulfation
Chondroitin sulfate and dermatan sulfate	D-glucuronic acid (GlcA) and *N*-acetylgalacotosamine (GalNAc); GlcA moiety of dermatan sulfate is epimerized into iduronic acid (IdoA)	→4) β-D-GlcA or α-L-ldoA (1→3) β-D-GalNAc (1→	Commonly C4, C6 on GalNAc, C2 on IdoA and rarely C3 on GlcA
Heparin and heparan sulfate	D-glucuronic acid (GlcA) and *N*-acetylglucosamine (GlcNAc); Epimerization of uronic acid into either GlcA or IdoA	→4) β-D-GlcA or α-L-IdoA (1→4) β-D-GlcNAc/S (1→	*N*-sulfation/acetylation as well as C2, C4, C6 and C3 on GlcNAc/S
Keratan sulfate	Galactose (Gal) and *N*-acetylglucosamine (GlcNAc)	→3) β-D-Gal (1→4) β-D-GlcNAc (1→	Only C6 sulfation on GlcNAc
Hyaluronic acid	GlcA and GlcNAc	→4) β-D-GlcA (1→3) β-D-GlcNAc (1→	Unsulfated

2.1.1. Chondroitin sulfate and dermatan sulfate

Chondroitin sulfate and DS contain the same amino sugar (GalNAc), but GlcA in CS undergoes epimerization to IdoA in DS. The CS is synthesized in the Golgi apparatus and attached to specific core proteins (Vynios, Karamanos and Tsiganos, 2002). The biosynthesis of CS begins with the formation of a tetra-saccharide linker xylose-galactose-galactose-GlcA on serine residues of the core protein. Next, GalNAc is added to the non-reducing GlcA by β1→4 linkage (Beaty and Mello, 1987). Chondroitin synthase sequentially adds GalNaC and GlcA to form a linear non-sulfated chondroitin. Then, chondroitin-backbone is further modified through the action of various sulfotransferases to produce different types of sulfated CS, while epimerization of GlcA into IdoA results in DS (Malmstrom and Aberg, 1982). CS consists of disaccharide units [→4 GlcA β1→3 GalNAc 1→] that are commonly sulfated at either the C-4 or C-6 position of GalNAc. The term CS-A has been used to describe CS rich with disaccharide units that sulfated at 4 (Δdi-4S) or 6 carbons (Δdi-6S) of their amino sugar. In addition, the disaccharides with different numbers and positions of sulfate groups vary among tissues and species. For example, CS from bovine tracheal cartilage and pig laryngeal is composed of monosulfate disaccharide units that are mainly sulfated at C-4. Nakano et al. (2012) reported that the predominant CS-disaccharide units found in chicken tissues are mainly Δdi-4S with some Δdi-6S. Chondroitin, a nonsulfated CS chain was found in the bovine cornea (Meyer and Linker, 1953), while disulfated disaccharide units of CS-D (6S of GalNAc and 2S of GlcA) and CS-E (both 4 and 6S of GalNAc) were found in CS extracted from shark cartilage (Suzuki, 1960) and squid cartilage (Kawai and Seno, 1966), respectively. Trisulfated disaccharides are a minor component of the CS chain in mammalian cartilage tissues, but they are found in large amounts in CS isolated from marine animals such as the fucosylated CS isolated form the sea cucumber (Shida, Mikami, Tamura and Kitagawa, 2017). Moreover,

variation also occurs in molecular weight. A range of molecular weight from 20-80 kDa were reported by Seikagaku Corp. (2009) from a number of commercial CS from whale and shark cartilage.

DS, on the other hand, also known as CS-B, has a hybrid copolymeric structure consisting of low modified CS and highly modified DS domains (Trowbridge and Gallo, 2002), which is commonly referred to as a CS/DS copolymer. The content of IdoA ranges from 0% in CS to 80-90% in various DS chains, were mostly found in PGs from animal skin tissues (Choi et al., 1989; Karamanos et al., 1995). Nakano et al., (1996) reported that pig skin CS/DS contains a high IdoA content accounting for approximately 80% of the total uronic acid. While in chicken comb CS/DS, an IdoA to GlcA ratio of approximately 3:7 was reported (Nakano and Sim, 1992). The degree of sulfation and molecular mass of CS/DS also varies depending on the tissue and animal species. DS obtained from the electric eel was composed of non-sulfated, mono-sulfated and disulfated disaccharides (Souza, Dellias, Melo and Silva, 2007). The occurrence of a disulfated DS unit has also been reported in porcine skin, while trisulfated DS units are found in Hagfish skin (Garnjanagoonchorn et al., 2007; Lauder, 2009). The molecular weight of the CS/DS copolymer chains are slightly less than the CS, where a range from 10-40 kDa are reported from different animal sources (Seikagaku Corp., 2009).

CS and DS have many important physiological functions. As the essential component of ECM of connective tissues, CS chains are suggested to be mostly responsible for the function and elasticity of the articular cartilage and are also involved in the bone growth process during the animal and human growth phase (Kuettner et al., 1991). Studies have demonstrated that the sulfation pattern of CS is related with cancer cell mechanisms, revealing its ability and potential role as a biomarker for early detection of diverse types of cancers (Pothacharoen et al., 2006).

Furthermore, the anti-infective role of human milk CS has also been reported (Dinglasan, et al., 2007; Coppa et al., 2013). On the other hand, DS, as the predominant GAG expressed in skin PGs, is an important component released during wound healing and may form complexes with heparin cofactor II to act as an anticoagulant (Trowbridge and Gallo, 2002). Both CS and DS have been implicated to have important biological functions during the processes of neural development in the brain such as the hemostasis of oxidative stress (Schiraldi, et al., 2010; Purushothaman, et al., 2007).

2.1.2. Heparin sulfate and heparin

Heparin and heparan sulfate share a similar biosynthetic pathway as CS and DS, which also begins with the formation of the tetra-saccharide linkage on serine residues of the PG. Next, a-*N*-acetyl glucosaminyl transferase adds GlcNAc instead of GalNAc to the non-reducing terminal GlcA. The GlcA residues may be epimerized into IdoA by C5-epimerase enzymes (Feyerabend et al., 2006). The glucosamine residues could be either *N*-deacetylated and *N*-sulfated by a family of *N*-deacetylase/*N*-sulfotransferase enzymes to form GlcNAc/S (Saribas et al., 2004). The C-6 and C-3 of GlcNAc and C-2 of IdoA or GlcA can also be *O*-sulfated by a group of O-sulfotransferases (Carlsson and Kjellen, 2012). Heparin has a structure similar to that of HS but displays a greater *N*- and *O*-sulfation and IdoA content, which also possesses the greatest negative charge density of any known biomolecule (Capila and Linhardt, 2002). Due to their complex structure properties, HP/HS exhibit diverse biological functions by interacting with other extracellular and cell membrane molecules such as thrombin/antithrombin (Onishi et al., 2015).

2.1.3. Hyaluronic acid

Hyaluronic acid, as the only non-sulfated GAG, is a linear unbranched polysaccharide composed entirely of alternating disaccharides units of GlcA and GlcNAc linked by β (1→3) and

β (1→4) glycosidic bonds. Hyaluronan synthase polymerizes the GlcNAc and GlcA repeating moieties to the growing HA carbon chain. This linear polysaccharide can reach molecular mass of 3000 to 8000 kDa, which is much larger than the sulfated GAGs (Stern et al., 2009). It has one carboxyl group per disaccharide repeating unit along the large chain that provides a very negatively charged polyelectrolyte at neutral pH. Therefore, it can hold a large number of water molecules in its molecular domain and occupies enormous hydrodynamic space in solution (Stern et al., 2007). This "swelling property" together with its chemical structure gives it a wide-ranging physicochemical and biological functions such as lubricity, viscoelasticity, biocompatibility, angiogenic effect during wound healing, and immune-stimulation (Gandni et al., 2008).

2.2. Current GAG sources and applications

In the last decade, an increasing number of GAG applications have been reported. Their commercial demands have been extended to different markets, such as pharmaceuticals, nutraceuticals as functional food ingredients, cosmetics and biomaterials for drug delivery and tissue engineering (Kovensky et al., 2017). For example, heparin has been an established anticoagulant drug for more than 60 years (Barrowcliffe, 2012). The average annual increase in heparin global sales is over 9% from 2011 to 2015 (Research QY., 2017a). The most successful commercial products of CS are those associated with cartilage regeneration, anti-inflammatory activity and osteoarthritis, while DS is in wound repair (Conte et al., 1995; Volpi, 2009). The CS market is expected to grow at the rate of 15% per year, reaching 3 million kg by 2021 (Research QY., 2017b). Other recent applications of CS and DS, in combination with other biopolymers such as HA and collagen, include the development of a biodegradable drug delivery vehicle and a biomaterial scaffold for tissue engineering (Xiong et al., 2012). HA is likely the most utilized GAG polymer in both medical and personal care applications to address physiological and pathological

changes that occur with aging. Due to its mucoadhesive behavior and ability to maintain the firmness of the skin cells as well as its soothing and antioxidant effects, HA is widely used in cosmetic products for skin (Stern et al., 2007). The global HA market is predicted to reach $10.8 billion by 2020 (Grand View Research Inc., 2015).

The types of applications for the formulations of GAG and GAG-derived products are dependent on their concentration and purity of each GAG as well as their origin. The current GAG sources for industrial scale production of commercially relevant GAG-containing products are mainly from animal and microbial sources.

2.2.1. Animal tissue GAGs

Traditionally, GAGs are obtained from mammalian tissues generated mainly from the slaughterhouse (Fu et al., 2016). Specific animal tissues have been used for GAG extraction, for example: (1) hyaluronan from rooster combs, bovine/porcine skin, eyes and umbilical cords, (2) chondroitin from animal cartilages, (3) dermatan sulfate from mucosal tissue and animal hide/skin, and (4) heparin from porcine/bovine mast cells that are found in intestine and lung tissues. Moreover, the marine and meat processing generate a significant amount of solid and liquid by-products, which can also be considered as valuable sources for GAG extraction, since they are cartilaginous rich materials (cartilage, skin, bones, tendons, etc.). Marine organisms like sponges, sea cucumbers, squids, mollusks, invertebrates and bony fishes are well-documented as potential raw materials for GAGs (Vázquez et al., 2013). Nakano et al. (2012) also reported a method for CS extraction and purification from the cartilage tissues present in meat processing by-products such as mechanical deboned meat. The GAGs from mammalian milk have been shown to positively influence the health of the newborns (Coppa et al., 2011). The peak secretion of GAGs is found in the colostrum compared to mature milk; however, the presence of colostrum in

commercial bovine milk is undesirable (Coppa et al., 2011; Scott et al., 2010). As a matter of fact, bovine colostrum is an agricultural by-product currently used as raw material for the production of milk oligosaccharides and immunoglobulin concentrates (Scott et al., 2010), which also could be a possible valuable raw material for milk GAG extraction.

However, concerns such as the presence of undesirable components (protein, peptides and nucleic acids), contamination risks (viruses and prions) and other food chain concerns have motivated the development of methods for industrial production of GAGs through alternative sources such as microbial fermentation and metabolic modified cell cultures (Fu et al., 2016).

2.2.2. Microbial production of GAGs

In order to avoid health and ecological problems derived from using mammalian and fishery wastes as substrate, different alternatives using the microbial production of GAG polymers have been reported (Schiraldi et al., 2010; Cimini, et al., 2012; Liu, et al., 2008). The non-sulfated GAGs are naturally produced by some bacteria, such as *Escherichia coli* K5, *E. coli* K4, *Pastuerella multicide* and Lancerfield group A &C *streptococci* as part of their mucoid capsule to facilitate their pathogenicity (Datta et al., 2017). In humans and other animals, the biosynthesis of GAGs is performed in three steps: firstly, the precursor synthesis, then the polymerization of the sugar moieties, finally, the post-polymerization modifications resulting in the complex GAG structures possessing multiple specific functional capacities. In microorganisms, however, GAG biosynthesis only consists of the first two steps without the last post-polymerization modifications due to the absence of certain sulfotransferases and epimerases (Badri et al. 2018). Thus, HA is the only GAG that does not undergo post-polymerization modifications and can be directly produced from a microbial source (mostly *Streptococci*) by fermentation (Schiraldi et al., 2010; Cimini et al., 2017). This fermentation generates the best yields with greater concentrations of HA (>3 g/L) at lower

costs and with more efficient downstream processes (Kim et al., 2006; Rangaswamy et al., 2008). In the past few years, the bacterial production of HA has been industrially developed to gradually replace the HA obtained from animal origin. The microbial production of chondroitin, the CS precursor, was successfully obtained by *E. coli* K4 under diverse experimental conditions and fermentation devices (Schiraldi et al., 2010). Here the chondrotin concentrations were increased to 3 g/L using a membrane bioreactor. Nevertheless, the chondroitin is unsulphated with a furanose residue, which needs a subsequent step of chemical sulfation and the hydrolysis of a fructose monomer (Bedini et al., 2011). The combined process of microbial fermentation and chemical sulfation is a complementary alternative that provides a more sustainable source for food-grade CS. Currently, due to the limitation of having no post-polymerization modification capability, the microbial system is mainly used for the production of chondroitin or heparosan, as the precursor stocks for the subsequent chemoenzymatic synthesis for animal-free chondroitin sulfate and heparin production (Vaidyanathan et al., 2017). The details on chemical and chemoenzymatic synthesis will be discussed later.

2.3. Production methods of GAG polysaccharides

Since the mainstream GAG production still relies on animal sources, the production methods have been optimized to involve four basic steps: (1) preparation of the tissue; (2) extraction of GAG from tissues; (3) recovery of raw GAGs; (4) purification of GAGs. Different compounds, including chemical solvent and detergents used during the production process, as well as the peptides, protein and nucleic acid from GAG containing tissues, are commonly contaminating the samples; hence reducing their commercial value and limiting their usage areas (Volpi, 2009). On the other hand, chemical and chemoenzymatic synthesis of GAG polysaccharides are rapidly advancing as future substitutes for conventional extraction methods. Currently, research groups

worldwide are working towards the development of both GAG production methods, the details of which are discussed in the following sections (Figure 2.2).

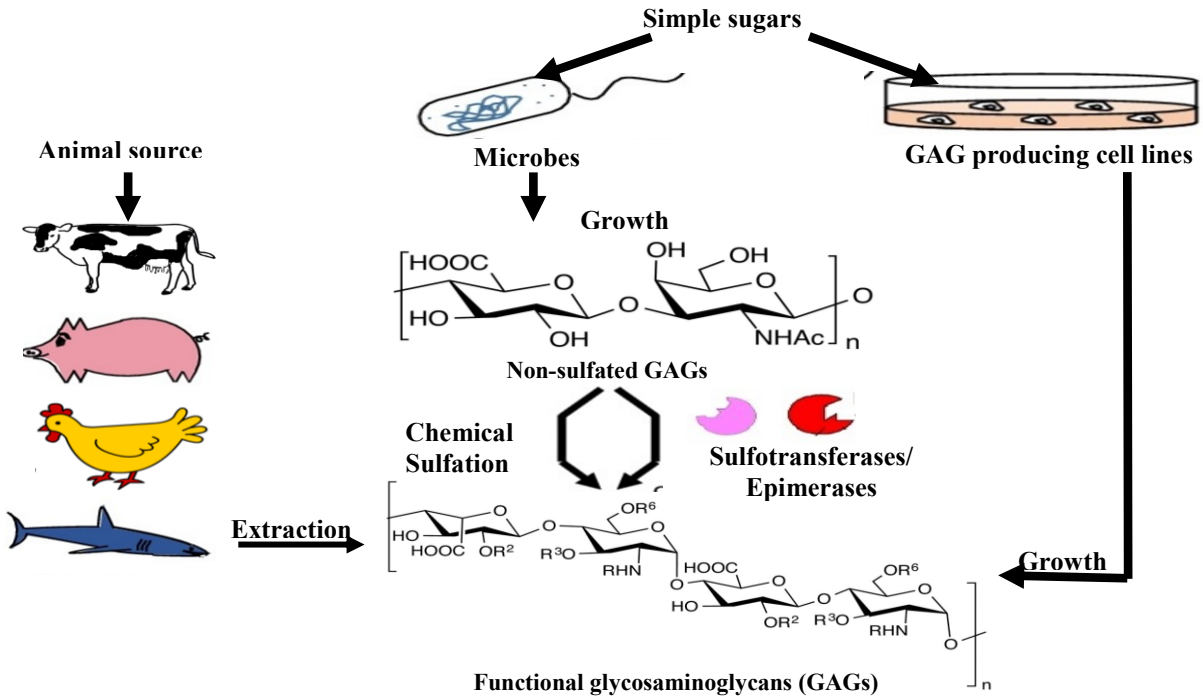

Figure 2. 2. Current production methods for GAGs. This figure is reproduced from Badri et al. (2018) with minor modifications with permission from Elsevier press.

2.3.1. Extraction and isolation of GAGs from animal and microbial source

Traditionally, GAGs are produced from mammalian tissues and shark fins. However, due to the concern of infectious diseases (i.e. bovine spongiform encephalopathy), religion issues, and other food chain issues, the exploration of using poultry and marine industry by-products as sources for GAGs has received increasing attention. Cartilaginous tissues from meat and marine by-products are the major sources of CS, while most DS and HA come from non-cartilaginous connective tissues (i.e. skin, tendon and skeletal muscle epimysium). In general, the methods for GAG isolation require various steps based on: (1) chemical hydrolysis of animal tissues; (1) breakdown of proteoglycan core (for sulfated GAGs); (3) recovery of GAGs; (4) purification of

GAGs. The first two stages are mostly conducted by alkaline hydrolysis at large concentrations of guanidine chloride or urea; subsequently there is selective precipitation of GAGs using cetylpyridinium chloride (CPC), potassium thiocyanate, or alcoholic solutions (Chascall, et al., 1994) and deproteinization by trichloroacetic acid. The final purification is usually done by gel filtration, ion-exchange and/or size-exclusion chromatography (Sumi et al., 2002).

Various extraction and isolation methods have been developed to replace the classical method for pursuing high yield and low-cost extraction processes and maintaining the quality and purity of GAGs. Figure 2.3 shows a flow chart representing all the potential steps described for the purification of animal GAGs. First, tissue samples are cut into small pieces. Large fat content tissues (i.e. skin) are defatted by first simple ethanol wash then centrifugation and/or filtration in the next step after fat is released from the tissue hydrolyzation. Subsequently, the pretreated tissue is hydrolyzed by proteases under controlled conditions. Purified papain is a powerful digestive enzyme and is commonly used to liberate whole GAGs from tissues at the laboratory bench scale. However, due to the large cost for double-crystalized papain enzyme, multiple enzymes have also been studied to achieve a more efficient breakdown of proteins and a good recovery of undamaged GAGs with minimal proteins/peptides attached. Nakano et al (2012) evaluated and compared the proteolytic activities from papain, pancreatin, kiwi fruits and Flavourzyme on the extraction of CS from boiler chicken biomass. The results showed that papain digestion was the most efficient followed by pancreatin digestion. At the same time, a two-step enzymatic processing with alcalase and Flavourzyme also showed good yield with high purity CS (Kim et al., 2012). The separation of hydrolysates is generally carried out by simple centrifugation to remove the GAG-rich supernatant from the undigested protein precipitate, which is then useful as substrate for animal/fish meal production.

The subsequent phase of alcoholic treatment is indicated by several studies as crucial for the recovery of extracted GAGs from the major soluble protein/peptides presents in the hydrolysate (Tadashi et al., 2006; Murado et al., 2010). The effectiveness of the process is dependent on the type and alcohol concentration, in some cases, the influence of temperature and time are also important (Murado et al., 2010; Nakano and Scott, 1989). Ethanol is the most commonly selected reagent for such precipitation due to its widespread use as a solvent intended for human contact or consumption. When using ethanol up to 75% in the presence of sodium or potassium acetate, all the GAGs could be precipitated. Nakano and Scott (1989) also reported that overnight setting at 4 °C could help the precipitation. The repetition of this procedure, under the same experimental conditions was also reported to increase the recovery of the GAGs.

The recovered GAGs are then fractionated by one of the following methods: ethanol fractionation (based on solubility in different concentrations of ethanol), anion exchange chromatography (based on binding affinity of GAGs with different concentrations of NaCl), or ultrafiltration/diafiltration (UF-DF based on different molecular weights). Nakano and Scott (1989) proposed to chromatograph bovine cartilage extracted GAGs on a DEAE-sephacel (Pharmacia-Biotech) anion exchange column to separate non-sulfated GAG (HA) from sulfated GAGs. Then, the sulfated GAGs could be fractionated at different final concentration of ethanol. Study showed that CS/DS copolymer could be precipitated with 18% ethanol, CS was recovered at ethanol concentration of 40%-50%, while keratan sulfate was obtained by 65% ethanol in the presence of 5% calcium acetate/0.5 M acetic acid (Nakano and Scott, 1989). The membrane technologies (i.e. UF-DF) have also be used in the last step of the purification because of easy scale-up, cost effectiveness, and simplicity of operation. However, the most effective way of using such technologies is still limited by removing salts (MW< 3 kDa) or separating HA (MW >300

kDa). Generally, GAG extraction from microbial sources is easier and involves using less chemicals than those reported from animal sources. Cellular biomass precipitation (centrifugation), deproteinization using proteases and membrane purification are the most conventional procedures.

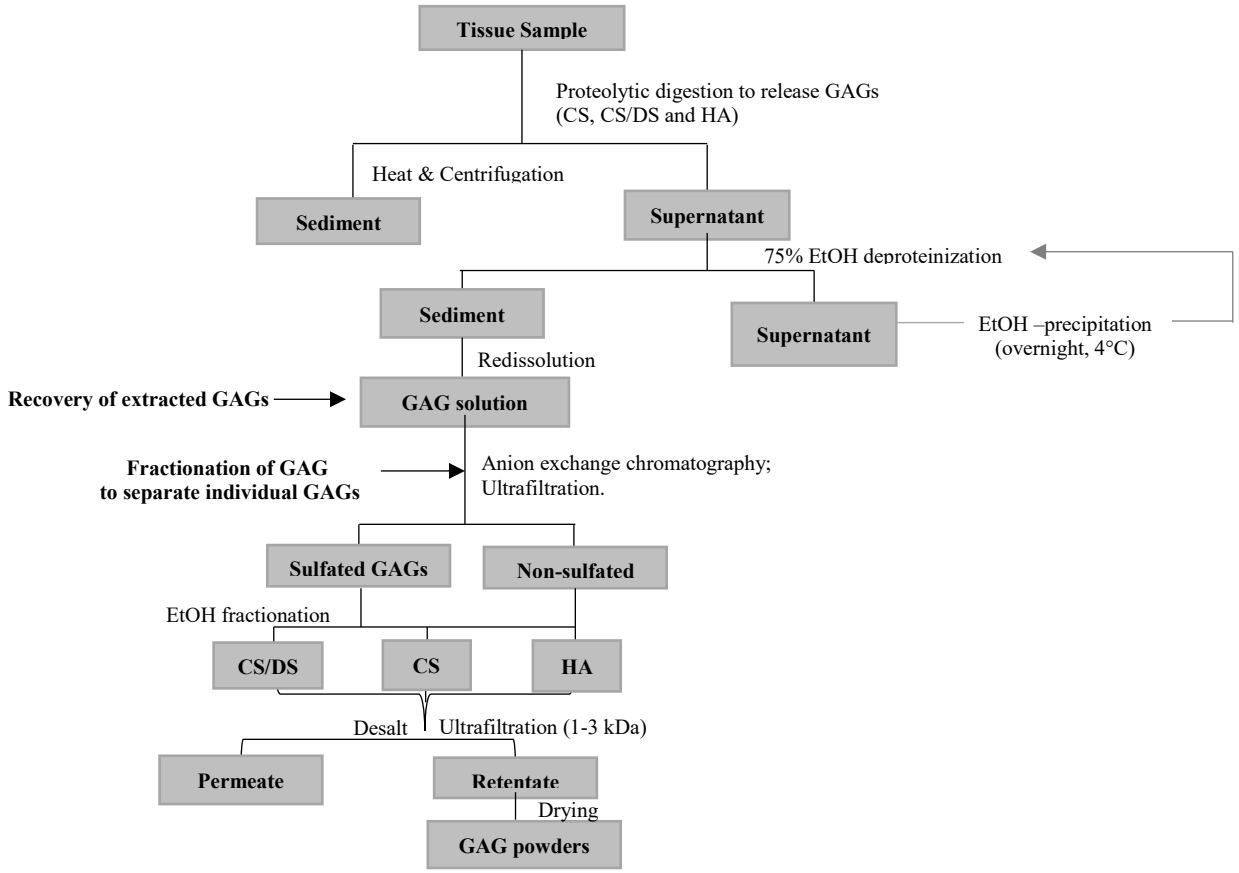

Figure 2. 3. Overview of food-grade GAG extraction and purification processes from animal tissues. This figure is reproduced from Wang and Betti (2017) and Nakano et al. (2012) with permissions from Elsevier press.

2.3.2. Chemical and chemoenzymatic synthesis of animal-free GAGs

Pharmaceutical applications demand highly concentrated and pure GAGs in comparison with cosmetic, dietary supplements or food ingredient applications. Heparin is one of the first natural biopolymeric drugs and also is one of the few carbohydrate drugs that is still currently in

widespread clinical use as an anticoagulant. The production of pharmaceutical heparin is estimated at almost 100 metric tonnes annually, with China being the largest supplier to meet more than half the world demand in 2016 (Tremblay, 2016). Commercial animal-sourced heparin production begins in non-cGMP animal slaughterhouses, followed by extraction and purification in cGMP facilities (Fu et al., 2016). However, concerns have risen from safety issues since the tissues collected in non-cGMP slaughterhouses harbor contamination risks from virus and prions. Indeed, this is even more so for the contamination from other types of GAGs since they are all co-existing in the ECM of animal tissues. The crisis of pharmaceutical heparin that was contaminated with oversulfated CS in 2008 led to 81 deaths and 785 reports of serious injuries associated with its use as a drug (Laurencin and Nair, 2008). This event necessitated the development of viable alternate sources for heparin production. In fact, the development of the chemically synthetic heparin was pioneered by Choay and colleagues in the late 1970's (Walenga et al., 2002). After two decades of their efforts, the first chemically synthesized ultra-low molecular weight heparin pentasaccharide was commercially produced by Arixtra^{TM} (Walenga et al., 2002). However, this chemical technique uses organic reactions and is very time consuming, extremely costly, and cannot be applied to make GAG polysaccharide (Walenga et al., 2002). Nowadays, with a better understating of the GAG biosynthesis pathway and the availability of key enzymes involved during the process, the current biochemical and metabolic technologies have provided strategies for producing complex GAG from a non-animal source. The chemo-enzymatic synthesis of CS and HP uses a microbial chondroitin and heparosan as the polysaccharide backbone and employs recombinantly microbial expressed sulfotransferases and epimerases to catalyze the post-polymerization modification (Badri et al., 2018). This method facilitates operating conditions, reduces the number of steps and increases the ability to synthesize large GAG polysaccharides.

Recently, the chemoenzymatic synthesis of non-toxic heparin dodecasaccharides could be achieved at the gram scale and is now ready for pre-clinical studies for the very first time (Xu et al., 2017). Although GAG production has a promising future, there are still many challenges to overcome to meet the world GAG demand through large-scale synthetic GAG production. Some of the many hurdles include the high cost of the process, metabolic engineering intracellular precursors for the process, and finding and selecting the best synthetic biology tools (Badri et al., 2018).

2.3.3. Characterization of GAGs

The difference among the various GAGs is attributed to their particular identity and arrangement of components, including uronic acid, amino sugars and sulfate patterns; thus, the molecular analysis of GAGs is the basis to understanding their structure-activity relationships. The content of uronic acid (both GlcA and IdoA) is determined using a modified method of the carbazole reaction of Kosakai and Yoshizawa (1979). The Elson-Morgan method (Boas, 1953) is a simple assay for the determination of the total content of amino sugars in a GAG hydrolysate. However, the hydrolysis at 100°C and acetylation usually demands intensive time and labor. More attention has been focused on ion exchange chromatography, high performance liquid chromatography (HPLC) with a reversed-phase column, gas chromatography and thin layer chromatography for glucosamine and galactosamine analysis (Studelska et al., 2006; Cheng and Kaplan, 2003). The main methods used for the assay of sulfate group include gravimetric estimation as barium sulphate, turbidimetry and ion chromatography (Wang et al., 2004). In contrast to turbidimetry, the advantages of ion chromatography are its simplicity, good reproducibility and sensitivity, and no interference by impurities. The content of the acetyl group in GAGs can be estimated by gas chromatography by analyzing the content of acetic acid

recovered from *N*-acetylhexosamine after hydrolysis in strong acid (Nakano et al., 2004). The molecular mass of GAGs is commonly determined by size exclusion HPLC and gel filtration chromatography.

The emerging structure identification methods of GAGs are electrophoretic analysis combined with enzymolysis technology (Ola et al., 2005). Cellulose acetate electrophoresis is the method widely used to identify GAGs (Nakano et al., 2010). The mobility of GAG on cellulose acetate is dependent mainly on its charge density and to a certain extent on its molecular mass. Based on the different buffer systems, CS, CS/DS, KS, HP/HS and HA are electrophoretically separated from each other (Seno et al., 2008; Nakano et al., 2010). The electrophoretically separated GAGs are localized by staining with Alcian blue (Nakano and Scott, 1989). Recently, capillary electrophoresis has been shown to be a useful method for quality control of CS or HS products in the pharmaceutical industry (Malavaki and Asimakopoulou, 2008). However, for the identification of GAGs extracted from a new source, their electrophoretic mobility may not correspond to the standard GAG due to the heterogenicity of the GAG structure among different species. Therefore, the identification of these new GAGs need further analyses by enzymatic methods. GAG lyases from bacterial sources (more recently of recombinant origin) can selectively degrade GAGs. Chondroitinase ABC can degrade virtually all CS, DS and HA, while leaving HP/HS and KS chains intact. Conversely, heparitinase enzymes can degrade nearly all forms of HP/HS, but are unable to degrade CS, HA, DS and KS (Hernáiz and Linhardt, 2001). Hyaluronidase from *Streptomyces hyalurolyticus* can degrade HA but no other sulfated GAGs (Ohya and Kaneko, 1970). More importantly, chondroitinase B only acts on the DS and chondroitinase C is specific for chondro-6-sulfate. After enzymatic digestion, the degradation of GAG is determined by electrophoresis. Moreover, the generated disaccharides/oligosaccharides containing a Δ4,5-

unsaturated uronic acid residue at the new non-reducing terminal end exhibits an absorbance maximum at 232 nm, which permits the detection of the degradation products by HPLC. Karamanos et al. (1994) developed an HPLC method with an amino column to identify 22 different unsaturated disaccharides derived from chondroitinase digested CS and DS with different degrees of sulfation. Anion exchange/ion-pairing HPLC have also been reported for the quantitative analysis of CS by the determination of unsaturated disaccharides released after enzymatic digestion (Nakano et al., 2010). Mass spectrometry (MS) has also been applied to the analysis of oligosaccharides obtained from various GAGs. It is comparably sensitive and has the particular advantage that individual GAGs can be easily differentiated by the characteristic mass differences of their polymeric repeating units. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS, ESI-MS is a suitable method for characterization of oligosaccharides. The LC-MS/MS techniques provide excellent mass accuracy, structural information and the ability to quantify the fragments. (Nimptsch et al., 2009; Jen and Leary, 2010; Seoa et al., 2011). Furthermore, a powerful analytical technique as LC-fluorescence-MS has been developed by Volpi et al. (2014) that takes advantage of a precolumn with fluorescently tagged disaccharides/oligosaccharides prepared from GAGs after their enzymatic breakdown. These advanced analytical methods have aided GAG structural analysis and improved insights into the GAG structure-activity relationships.

2.4. Depolymerisation methods for GAG polysaccharides

In the study of structure-activity relationships of polysaccharides, researchers found that their bioactivities are related to the types of glycosidic bonds they possess, their monosaccharide compositions, their solubility, the identity of their functional groups, and their relative molecular masses. The first two factors (glycosidic bonds and monosaccharides composition) are the inherent

properties of the polysaccharide and as such are unable to be modified; however, the rest of the factors are variables that can affect the nature of their bioactivities and can be changed by using proper modification methods. Because GAGs are soluble bioactive molecules with functional groups (carboxylic and sulfate groups), their molecular modifications are mainly related to their molecular mass. When the molecular weight is too large, it is less able to penetrate cell membrane barriers to exert its pharmacological effects (Alban and Franz, 2000). For instance, CS polysaccharides present have an absorption problem in the digestive system when orally administered. Low molecular weight HP (LMWH), with a molecular weight between 4-10 kDa, has better anticoagulant activity when compared to the native HP (Fu, Suflita and Linhardt, 2014). The depolymerisation of GAG polysaccharides can be achieved by enzymatic (biological) and non-enzymatic (chemical and physical) means to obtain numerous structural derivatives (Figure 2.4). Through appropriate methods, structural changes can be tailored to achieve specific physicochemical properties and bioactivities.

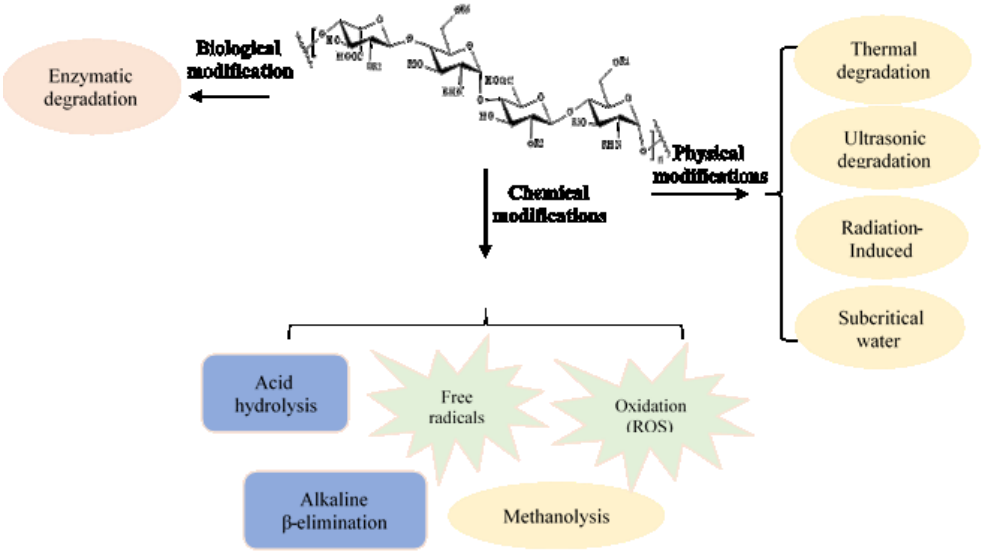

Figure 2. 4. Major methods of depolymerisation of GAGs. The figure is reproduced from Stern et al (2007) and Li et al (2017).

2.4.1. Enzymatic depolyermisation of GAG polysaccharides

In general, the biological modification of GAGs mainly refers to enzymatic modification, which is the catalysis of polysaccharides with AG lyase. The advantages of using the enzymatic method is that it has both good specificity and efficiency and has few side effects. The molecular masses of these GAG products are relatively uniform after enzymatic degradation and do not significantly influence the side chains (i.e. sulfate groups) of the GAG polysaccharide. The main effect of enzymatic degradation is to degrade the backbone of polysaccharides, leading to a reduction of the molecular weight and reduction of its viscosity (Oosterveld et al., 2002; Mahammad and Khan, 2004; Stern et al., 2007). As discussed in the previous sections, most GAG lyases are very specific for each type of the GAG. LMWHs can be produced by microbial degradation by a bacterial enzyme (heparinase I) through enzymatic β-elimination cleavage. Chondroitinases are often used to degrade CS and DS polysaccharides but tend to completely digest glycosaminoglycans into disaccharide units, which then do not exert any bioactivity (Stern et al., 2007). Smaller GAG fragments, in the range between tetra-saccharides to oligosaccharides, have a suppressing effect on apoptosis by inducing heat shock proteins. This effect is not seen with disaccharides (Xu et al., 2002). On the other hand, the testicular hyaluronidase does not have an absolute substrate specificity, thus has the ability to utilize chondroitin and CS as substrates as well. Gibson and Pearson (1982) used this enzyme to detect GlcA residues in CS/DS copolymers extracted from bovine periodontal ligament. Currently, its usage for enzymatic depolymerisation is limited to the laboratory bench scale for GAG-derived oligosaccharides because of its low-throughput and excessive expense for the mass production.

2.4.2. Non-enzymatic depolyermisation of GAG polysaccharides

Due to a consistent large demand for pharmaceutical grade LMWHs and an increasing demand for GAGs as biomaterials in medicine, cosmetics and food supplements, numerous non-enzymatic processes are emerging for creating depolymerised GAG polysaccharides involving the use of harsh chemical and physical modifications.

Similar to other polysaccharides, GAGs can be depolymerised by acid or alkaline hydrolysis (Inoue and Nagsawa, 1985). Specifically, glycosidic bonds of polysaccharides are cleaved by acid or alkaline solution, thus degrading polysaccharides into lower molecular weight fragments. However, chemical hydrolysis proceeds in a random fashion and produces a mixture of oligo- di- and monosaccharides instead of relatively unified molecular mass produced by enzymatic depolymerisation (Stern et al., 2007). The concentration of the acid/alkaline solution, reaction temperature and time are all key to control and obtain depolymerised products with different molecular weights. The stability studies of HP and CS with elevated temperatures under both acid and alkaline conditions were reported by Jandik et al. (1996) and Volpi et al. (1999). A decomposition pathway for HP and CS under these stressed conditions has been proposed. Recently, Lu et al. (2017) identified eleven di-, tri- and tetra-saccharides with or without sulfate/acetyl groups that are released from CS treated with trifluoroacetic acid at 100°C for 1 h. The study indicated that acid hydrolysis through hydrolytic cleavage could provide information on the structural alterations of CS-polysaccharides and produce CS-oligosaccharides. On the other hand, alkaline depolymerisation is based on the β-elimination cleavage of the glycosidic bond of amino sugar. The carboxyl group of the uronic acid residue needs to be chemically esterified in order to accelerate the process due to the surprising stability of heparin under alkaline conditions.

Several commercial LMWHs, such as Enoxaparin and Tinzaparin, have been obtained by a controlled chemical β-eliminative depolymerisation (Hirsh and Levine, 1992).

The oxidative depolymerisation relying on metal ion catalysis (Cu^{2+}/O_2) has been used to prepare low-molecular weight GAGs since reactive oxygen species (ROS) are involved in the degradation of essential tissues or related components including GAGs (Kogan et al., 2008). Of these oxidative methods, only hydrogen peroxide has been utilized to commercially prepare LMWHs for clinical use (Datta and Linhardt, 2017). The depolymerisation of other GAGs such as CS, DS and HA using free-radical depolymerisation or oxidative-induced deamination cleavage are still under evaluation. Most studies reported that these resulting fragments are not readily labeled with a UV tag to facilitate their purification and structural characterization (Gao et al., 2015). Toida and others (2009) also attempted to prepare chondro/dermato oligosaccharides through a methanolysis (solvolysis) method that can be characterized by both ^{1}H NMR and MALDI-TOFMS; however, all the internal repeating disaccharide units were desulfonated.

The depolymerisation of GAG polysaccharides can also be achieved by physical modification. The most commonly used methods are ultrasonic disruption, radiation-induced reaction and microwave exposure. Unlike the acid/alkaline hydrolysis, ultrasonication degrades GAG polysaccharides in a non-random fashion, resulting in a bimodal molar mass distribution (Vercruysse et al., 1995). This means that no matter how the polysaccharides' molecular weights were distributed before the treatment, the derivative products' molecular weight distribution was in a narrow range (Zhong et al., 2015). Dřímalová et al. (2005) compared ultrasonic degradation of HA with the gamma-radiation and microwave method. The results suggested that ultrasonic degradation is the preferable method since it is able to efficiently reduce molar mass down to 100 kDa without significant effecting the side chains (i.e. sulphate group) of the primary structure.

More recently, a subcritical water microreaction system has been suggested by Yamada et al. (2013) for CS depolymerisation. The results showed that no significant desulfation existed in the resulting CS oligosaccharides, and the authors recommended this method for the mass production of CS oligosaccharides.

As indicated above, both enzymatic and non-enzymatic depolymerisation processes affect the primary chemical structure of GAG polysaccharides by producing fragments containing double bonds or opened sugar rings. These altered molecules could be recognized by human immune cells as "foreign", and an immune reaction might be triggered by such modified biomaterials (Stern et al. 2007). The biocompatibility and bioactivities of these depolymerized GAG-derived oligosaccharides need to be carefully examined.

2.5. Antioxidant properties of GAGs polysaccharides

Oxygen is essential for normal life of aerobic organisms. Due to its large redox potential, oxygen is inevitably involved in the production of reactive oxygen species (ROS) such as superoxide anion, hydroxyl radical and hydrogen peroxide (Apel and Hirt, 2004; Weydert and Cullen, 2010). Studies indicate that low levels of ROS are essential in a variety of cellular functions including signal transduction and regulation of enzyme activity (Sauer et al., 2001). On the other hand, excessive ROS and free radicals could break the redox hemostasis and result in oxidative stress of cells (Ghosh and Myers 1998; Apel and Hirt, 2004). Normally, iron in the body is bound to storage or transport proteins and strictly regulated by enzymes. However, under conditions where there is excess iron, trace amounts of unprotected free iron may interact with oxygen and its derivatives via the Haber-Weiss reaction (Figure 2.5) and give rise to the very reactive and toxic hydroxyl radical (Halliwell, 1993).

$$Fe^{3+} + O_2^{\cdot -} \rightarrow Fe^{2+} + O_2$$

$$2O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2$$

$$Fe^{2+} + H_2O_2 \rightarrow \cdot OH + OH^- + Fe^{3+}$$

Figure 2. 5. The Haber-Weiss reaction (Haber and Weiss., 1932).

The catalytic effect of this transition metal ion can be reduced by molecules possessing chelating activity (Minotti, 1989). The reactivity of iron varies greatly depending on the type of ligand the chelator possesses. Generally, chelators with oxygen ligands such as citrate, prefer Fe^{3+}, thus decreasing the reduction potential of iron (Miller, Buettner &Aust, 1990). Whereas chelators that contain nitrogen ligands, such as phenanthrolines, inhibit the oxidation of Fe^{2+}. The most commonly used chelator in the food industry is ethylenediaminetetraacetic acid (EDTA), which is able to bind both Fe^{2+} and Fe^{3+}. In this case, the stability of the Fe^{3+} -chelator complex is found to be much greater than the binding to Fe^{2+} (Campo et al., 2006). Under normal circumstances, the body's antioxidant defense system is intended to minimize the production of these radicals and the subsequent damage they cause to biomolecules. However, when this system is compromised and unable to neutralize the excessive ROS, a fraction of ROS may escape this intrinsic clearance machinery and an extrinsic source of antioxidants are needed to restore the redox homeostasis (Birben et al., 2012; Gupta et al., 2014). Dietary antioxidant supplements are used synergistically with the enzymatic antioxidants to protect cells from oxidation (Thomas 1999) (Table 2.2). A lower antioxidant status indicates the antioxidant enzymes and dietary factors described above have failed to convert some harmful ROS into harmless compounds. The free radicals can initiate lipid peroxidation and protein oxidation (McCord, 1993). Low-density lipoprotein (LDL) peroxidation is a marker for cardiovascular diseases (Berliner and Heinecke 1996) and relate to

the role oxidized LDL plays in atherogenes. Recent studies have revealed that ROS-induced peroxidation products can damage the neural cellular components that can contribute to the development and progression of many neurodegenerative diseases (NDD) such as Alzheimer's disease (AD) and Parkinson's disease (PD) (Melo et al., 2011; Sultana et al., 2013). Examples of such oxidizing agents are malondialdehyde (MDA), a lipid peroxidation product, and the carbonyl groups generated during protein oxidation.

Table 2. 2. Antioxidant defense systems. The table is reproduced from Huang et al (2005).

Enzymes:	
	superoxide dismutase - convert radicals to hydrogen peroxide
	catalase - detoxifies hydrogen peroxide
	glutathione peroxidase - detoxifies hydrogen peroxide and lipid peroxidases in the presence of reduce glutathione
Dietary:	α-tocopherol – scavenges peroxyl radical intermediates
	ascorbic acid – water soluble antioxidant; acts synergistically with tocopherol
	β-carotene – quenching singlet oxygen and scavenging free radical species
	coenzyme Q10 – antioxidant enzyme cofactors
	EDTA – transition metal chelators

In addition, many biological molecules also have the ability to directly and indirectly act against oxidative cellular damage by different mechanisms, such as by directly scavenging active free radicals, by suppressing the formation of ROS, by reducing hydroperoxides and H_2O_2, and by sequestering metal ions (Figure 2.6). These systems act to aid repairing and/or clearing oxidative damage, and besides this, some may even induce the biosynthesis of other antioxidants or defense enzymes (Campo et al., 2006). Indeed, GAGs themselves have attracted increasing attention from many research groups due to their antioxidant potential and pharmacokinetic activities. The ability of acid GAG polysaccharides to bind other positively charged molecules and metal cations has been widely known and extensively studied, especially for cations such as calcium, copper and

also iron. Acid GAGs contain both carboxylic and ester sulphate groups. The studies showed that Ca^{2+}, Cu^{2+} and Fe^{2+} ions all bind both groups in CS sulfated at the C4 position (C4S) but such binding does not happen to CS sulfated at C6 position (Cael et al., 1978; Balt et al., 1983; Yang et al., 1986; Scott, Chen and Brass, 1992). This can be explained since all the major substituents of the disaccharide sugar rings are equatorial in C4S, except the axial sulfate ester group on position C4 of GalNAc (Cael et al., 1978). The position of the sulfate groups in C4S is along the centerline of the polymer backbone, which donates a large negative charge density to the molecule, so C4S does not self-aggregate. C6S, on the other hand, has sulfate groups at the periphery of the molecule, which can self-aggregate (Scott et al., 1992).

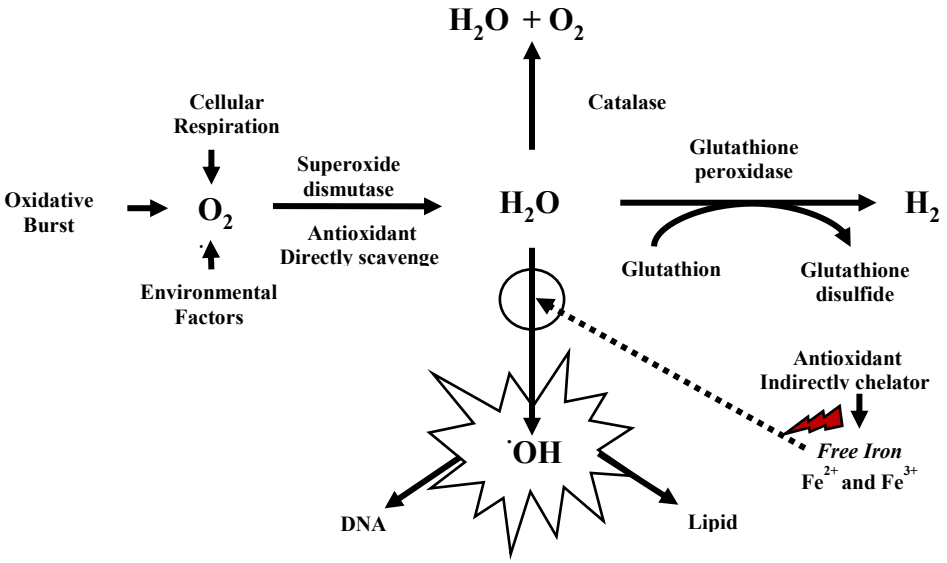

Figure 2. 6. Oxidative stress: role of antioxidant and free iron. This graph is reproduced from Young and Woodside. (2001) with permission from PMC press.

Many *in vitro* and *in vivo* studies showed that GAGs may protect endothelial cells from ROS induced oxidations. Okamoto et al. (1994) showed that CS proteoglycans have a neuroprotective activity against excitotoxic neuron cell death in primary cultured neurons of rat cortex. The antioxidant effect of various GAGs of different origins on Cu^{2+} were evaluated by Volpi and Tarugi (1999). Here hyaluronan had no effect, while CS from beef trachea produced a very strong

protective effect on LDL oxidation. Albertini et al. (1996) showed that CS sulphated at C4 (C4S) but not at C6 (C6S) can be inhibited by copper-induced LDL oxidation. C4S displays a better antioxidant effect than C6S and HA, which can be explained by the different sulfated group position as described before, where HA lacks the sulfate ester group. The carboxylic groups in both HA and CS can bind cations in any case, thus the larger antioxidant activity of C4S was suggested due to the additional contribution from the sulfate group at C4, which stabilizes the cation binding (Campo et al., 2006). Although most of the data suggested that the characteristics of the CS molecules (i.e. COO^- and SO_3^-) limited their antioxidant mechanism to chelation activity (Karlsson et al. 1988), Arai et al (1999) proposed that the decomposition of GAG by ROS produced neutralising molecules that in turn can act as direct radical scavengers that stop the chain of oxidation reactions on biomolecules.

2.5.1. Types of in vitro and in vivo antioxidant assays

A number of screening assays, both *in vitro* and *in vivo*, have been developed to investigate the antioxidant property of samples (diets, plant/meat extracts, commercial antioxidants etc.) and to identify and assess potential antioxidants that would be useful for food and biological systems.

In general, *in vitro* antioxidant tests using free radical traps are relatively straightforward to perform. Among free radical scavenging methods, the DPPH method is rapid, simple and inexpensive in comparison to other test models (Alam et al., 2013). Hydrogen peroxide and superoxide radical scavenging assays are used to measure ROS, and both of these are involved in the production of hydroxyl radicals that can lead to the initiation of lipid peroxidation. Hydroxyl radical scavenging activity can also be directly evaluated by the method of Kunchandy and Rao (1990). As for determining antioxidant activity against transition metals, hydroxyl radical scavenging capacity and metal chelating activity are often used as colorimetric methods that

measure the metal-chelating activity of an antioxidant with Cu^{2+} and Fe^{2+}. Antioxidant activity can also be evaluated by ferric (Fe^{3+}) reducing activity methods that are commonly used for detecting the ability of a tested compound to transfer electrons, resulting in a color change when Fe^{3+} is reduced (Wang et al., 2015)

For all *in vivo* methods, the samples that are to be tested are usually administered to the test animals (mice, rats, etc.) at a defined dosage for a specified period of time as described by each particular method. The animals are usually sacrificed and blood or tissues are used for the assays. Among all the *in vivo* assays, lipid peroxidation, superoxide dismutase, catalase and reduced glutathione estimation are the most frequently used (Alam et al., 2013).

Antioxidant activity usually cannot be determined based on a single antioxidant test model. Generally, in practice several *in vitro* test procedures are performed as a preliminary screening of the antioxidant activity for the samples of interest. Therefore, it is difficult to compare fully one method to another one. The researcher must critically verify methods of analysis before adopting a given one for research purposes.

2.6. The current and potential therapeutic use of GAGs polysaccharides

A mixture of HA and CS (Viscoat) has been used to limit eye tissue damage from oxidative attack (Takahashi et al., 2002), despite the difference in their antioxidant capacities. The antioxidant and anti-inflammatory effects of CS are being used to treat osteoarthritis (OA). Multiple controlled clinical trials have reported clinical benefits of CS (800-1200 mg) to reduce pain, joint swelling and effusion with an excellent safety profile (Uebelhart et al., 1998; Jackson et al., 2010). In fact, CS has been classified as a symptomatic slow acting drug (SYSADOA) and has been suggested as such by the Osteoarthritis Research Society International (OARSI) and the

European League Against Rheumatism (EULAR) in their latest recommendation guidelines (Zhang et al., 2008). However, the symptomatic effectiveness of CS for the osteoarthritic joint is still controversial and CS studies have sometimes been criticized for small sample size or short length of therapy. The molecular weight of CS also raises doubts toward its effectiveness when it is administrated orally. Since there are no specific glycosidases and sulfatases present in the stomach and intestinal wall for the CS digestion, the intact CS is found to have poor permeability through the gastric and intestinal mucosa (Baici et al., 1992). In order to exert its antioxidant or biological effects, the compound must be able to reach the target sites at a sufficient concentration (Campo et al., 2006). Therefore, a key property for CS to be bioactive is its ability to be efficiently absorbed from the gastrointestinal tract thereby gaining access to the desired target sites such as the blood, liver and infected joints. In a recently published study, a series of low molecular weight CSs (LMWCSs) were prepared from different sources. Both *in vitro* and *in vivo* results showed that LMWCS from shark cartilages protect chondrocytes from cell death and could be used for attenuating OA through regulating the complement system (Li et al., 2016).

Moreover, the evidence has revealed that antioxidant CS may also have therapeutic applications in diseases that are related to the central nervous system (CNS) such as AD and PD. These are neurodegenerative diseases found among the elderly and are likely induced or exacerbated by oxidative stress. CS-PGs are the most abundant type of PG found in the mammalian CNS, especially in perineuronal nets (a specialized forms of ECM). They mainly act as barrier molecules that affect cell migration and brain plasticity, particularly through their GAG chains. These chains form very negatively charged structures that can contribute to reduced local oxidative stress by scavenging and binding redox-active iron, thus providing neuroprotection to the net-associated neurons (Egea et al., 2010). On the other hand, abnormal protein aggregation is known to be a

common pathological biomarker of late-onset NDDs. These protein aggregates, including amyloid-β peptide (Aβ) aggregates in AD, can induce neuronal damage by inducing oxidative stress (Ariga et al., 2010). In an animal study, the process of Aβ aggregation can be enhanced by sulfated GAGs (i.e. HP) due to their binding to Aβ proteins through sulphate moieties (Zhou and Jin., 2016). However, the sulfated GAG-oligosaccharides or analogues may prevent Aβ aggregation by blocking β-sheet formation and inhibiting fibril formation (Zhou and Jin., 2016; Urbányi et al., 2005). These findings also reinforce the concept that the molecular weight of these sulfated polysaccharides can significantly affect their biological activities, ranging from a harmful effect to a potential therapeutic benefit.

Furthermore, the dynamic influx of dietary GAGs within the intestinal mucosa has been identified as an important factor shaping the ecophysiology of the gut microbiota (Koropatkin et al., 2012). Some probiotics such as bifidobacterial possess specific enzymes that can metabolize complex carbohydrates as a nutritional source (Ventura et al., 2007). It is reasonable to speculate that the carbohydrate fraction of GAGs could behave as prebiotics with bifidogenic effect since they do resemble oligosaccharides with a proven prebiotic capability to *Bifidobacterium bifidum* (Turroni et al., 2010). It is noteworthy that genome analyses of *Bifidobacterium bifidum* PRL2010 isolated from breastfed infants' stool revealed a nutrient-acquisition strategy that involves a host-derived glycan (Turroni et al., 2010). Coppa et al. (2013) had proposed that human milk GAGs could be a bioactive substance that benefit the breastfed newborn since they have multiple biological activities such as antiviral, antioxidant and bifidogenic effect. Although it seems quite promising for GAGs to have this probiotic effect, the undigested GAGs reaching the colon are expected to be high molecular weight polysaccharides ranging from 20 (HP) up to 3000 (HA) kDa. Based on the data from current studies, the carbohydrates with a proven bifidogenic effect are

fructo- and galacto-oligosaccharides identified in human milk (Clemens et al., 2014). It is possible that undigested GAG polysaccharides may not be utilized by the colonic bifido-bacteria. Gibson and Wang (1994) in their study on bifidogenic properties of different types of fructo-oligosaccharides stated that the growth of each of the different species largely depends on the type of oligosaccharides. The degree of polymerization (DP) of 4 had a greater bifidogenic effect than high molecular weight carbohydrates (13 DP) and branched chain varieties. There are only a few studies (Turroni et al., 2010; Coppa et al., 2013; Clemens et al., 2014) that have mentioned the possible bifidogenic effects of milk GAGs. Based on the previous discussion, the molecular weight of GAGs and their derivatives may also be important for their bifidogenic effect and requires further studies.

There has been an interesting connection made between the oligosaccharide prebiotic and iron absorption by Yeung et al (2006). However, it is not the first time that the undigestible carbohydrate has been related to the iron absorption. CS could directly interact with both the Fe^{2+} and Fe^{3+} forms of iron due to the particular chemical structure of CS, and its effect on non-heme absorption and iron absorption in humans are reviewed in the next section.

2.6.1. Antioxidant CS and iron deficiency anemia

Deficiency of iron and its associated anemia affect ¼ of the population worldwide (Mclean et al., 2008). Iron deficiency anemia (IDA) accounts for 50% of all anaemias with infants, children and women of childbearing age and are especially at risk in developing countries. As the lifespan of the population on the whole is increasing, the elderly become another group at risk of IDA since degenerative aging may lead to impaired iron absorption. In addition, people with restrictive diets (vegan) or chronic gastrointestinal inflammation, such as colitis and irritable bowel syndrome, are also at high risk. Therefore, IDA has become a prevalent micronutrient deficiency in both

developing and developed countries and represents both significant health problems and economic costs.

The antianemic effect of CS was not new to the scientific world. Due to its ability to bind iron, the CS-iron complex (Condrofer) has been used in the treatment of IDA. In a dated study, a complex of CS-Ferric (Condrofer) was given to experimentally anaemia-induced rats for 4 weeks to cause a more complete reversal of anaemia in the rats that were treated with CS-iron complex compared to iron alone (Barone et al., 1988). The authors suggested that this beneficial effect was probably due to the greater bioavailability of iron associated with this CS binding complex. However, when Allegra et al. (1991) compared the effectiveness of Fe-ferritin, CS-Fe complex and Fe-gluconate-Na as iron therapy to patients with iron deficiency for 6 months, the rate of positive responses from the patients receiving CS-Fe was less than that of Fe-gluconate-Na group. In a more recent study reported by Storcksdieck et al. (2006), no enhancing effect of purified CS and HA was observed for the non-heme iron absorption for young women receiving a semisynthetic diet prepared with hydrolyzed corn starch, chicken egg albumin and corn oil. The high molecular weights of the commercially purified CS and HA was suggested to be the main issue causing poor permeability by the enterocyte cells and thus poor bioavailability.

GAGs have been identified and extracted from different animal and bacteria sources (as described in the previous sections). GAGs can also be obtained through diets containing meat and milk. Ingestion of food has been shown to increase the plasma HA concentration to 13 times the basal levels (Fraser and Gibson, 2005). However, the capacity of digestive enzymes to hydrolyze bonded sulfated groups in GAGs has not been discussed, nor has the possibility of the consequences of alterations to the GAG structure during gastrointestinal digestion. The *in vitro* data suggests that dietary GAGs released from their protein core by gastrointestinal digestion may

play a role to enhance non-heme iron absorption and thereby improving nutritional iron status (Huh et al., 2004). Before giving an overview of these current efforts, the basic concepts on iron absorption/bioavailability will be introduced next.

2.6.2. Iron bioavailability

Nutritional iron deficiency is a state of imbalance when the iron content of the diet is insufficient to meet physiological needs. Iron is present in a wide variety of foods. Depending on the presence of dietary enhancers and inhibitors, non-heme (inorganic) iron absorption from the diet ranges between 1% and 15% (Fairbanks, 1998). Therefore, the cause of nutritional deficiency is often related to poor iron bioavailability rather than inadequate intake.

Iron bioavailability is defined as the proportion of the ingested iron that is absorbed and utilized for normal physiological functions and storage (Brabin et al., 2001). Iron in the diet comes in two different forms, namely heme and non-heme iron. Heme iron comes from animal sources whereas non-heme iron is found in both plant and animal sources (Fairbanks, 1998). Non-heme iron represents the largest fraction of total dietary iron, but its absorption rate may be only 1 to 7% from vegetable staples such as rice, wheat bran, and maize when consumed alone (Bothwell et al. 1979). Heme iron is from hemoglobin and myoglobin and is better absorbed than non-heme iron. In adults with adequate iron stores, approximately 25% of heme iron is absorbed (West et al., 2000). As non-heme iron in foods primarily exists in the insoluble ferric valence, apical non-heme iron uptake can be divided into two sequential steps: reduction of ferric iron to ferrous iron and apical uptake of ferrous iron. The overall iron acquisition pathway in the brush border of enterocytes involves both a ferrireductase such as DcytB and a ferrous iron transporter, DMT1 (Fleming et al. 1999). The pathway for heme iron absorption differs from that for non-heme iron. Heme iron is absorbed by receptor mediated endocytosis (Fleming et al. 1999). Once iron reaches the interior

of the serosal wall the iron is released as Fe^{2+}, oxidized by ceruloplasmin to Fe^{3+}, taken up by transferrin/paraferritin in the subendothelial capillary network for transport to the liver and then to the rest of the body (Figure 2.7).

2.6.3. Dietary enhancers o nonheme iron bioavailability

Although absorption from the non-heme iron constitutes a major fraction of daily iron intake, unlike heme iron, its absorption depends on the presence of a variety of dietary components ingested simultaneously (Reddy et al., 1996; Fleming et al., 1998). Ascorbic acid and muscle tissue from meat, fish, and poultry (Bjom-Rasmussen and Hallberg, 1979) enhance non-heme iron absorption, whereas phytic acid, polyphenols, and calcium salts (Gillooly et al., 1983; Lynch et al., 1994;) inhibit non-heme iron absorption. The mixture of foods eaten by humans, the interplay of nutrients and non-nutrients affects non-heme iron absorption in complex ways.

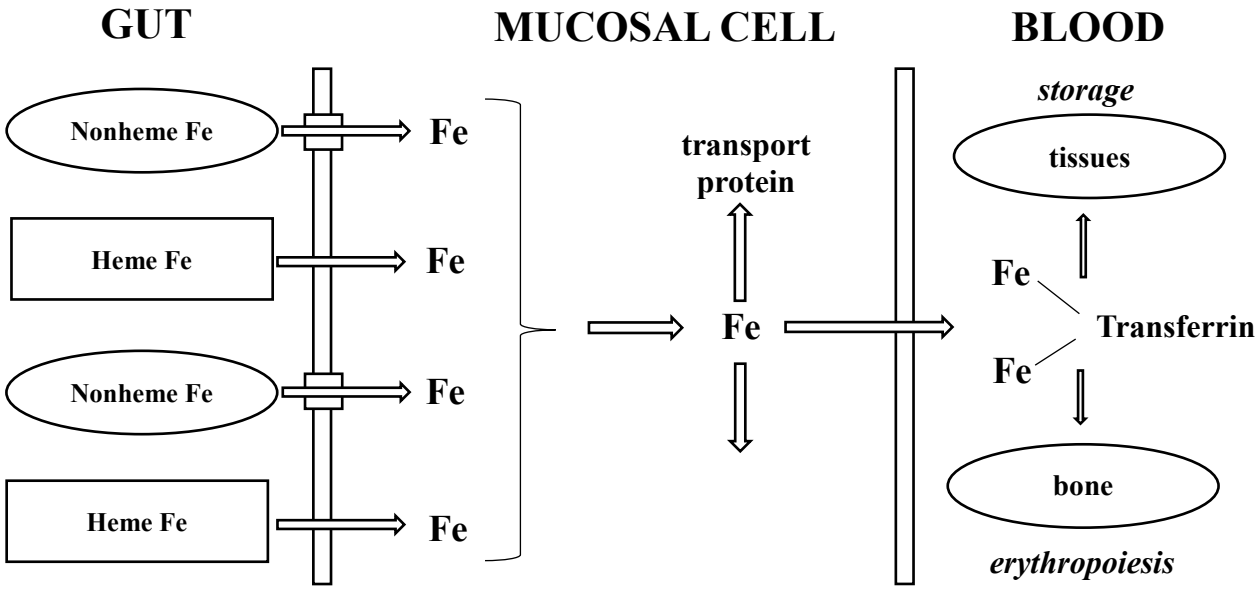

Figure 2. 7. Overview of iron absorption. The figure is reproduced from Uchida, (1995).

Meat offers not only very absorbable heme iron, but also is known to enhance dietary non-heme iron absorption. The unique components of animal tissues that enhance intestinal iron absorption are coined as the "meat factor". It was hypothesized that the meat factor prevents iron from polymerizing (precipitation) and maintains iron in a stable soluble form suitable for absorption (Carpenter and Mahoney, 1992; Hurrell et al., 2006; Li, Jiang and Huang, 2017). Meat proteins, as the main component in animal tissues, have been systematically studied regarding their effect on non-heme iron absorption by researchers all over the world for the last 30 years (Li, Jiang and Huang, 2017). Based on abundant data from both *in vitro* and *in vivo* studies, the following conclusions can be drawn: 1) The effect of meat protein on non-heme iron absorption is source dependent but not quality and quantity dependent. Animal tissue proteins such as beef, pork and chicken have enhancing effects, whereas eggs and ovalbumin proteins do not. 2) The Fe enhancing effect of meat proteins is amino acid specific, where a large concentration of histidine and lysine favor this effect. 3) The iron chelating and solubilizing peptides (histidine, lysine and cysteine) can be released by proteolytic digestion from the gastrointestinal tract.

However, findings also indicate that the factor in meat responsible for enhanced non-heme iron absorption may not be released during gastrointesinal digestion (Au and Reddy 1997). It may indicate that meat contains enhancing factors, capable of solubilizing iron, that are independent of proteolytic digestion (Au and Reddy 1997). A clear understanding of the positive effect of this meat factor has not yet been realized, but particular proteins and/or their constituents in meat that interact with non-heme iron to maintain its solubility are suggested to be responsible for the meat factor.

On the other hand, human milk, as the main food source in early life, is suggested to possess certain factors that enhance iron absorption in infants. Bovine milk, on the contrary, performs very

differently in terms of non-heme iron absorption. Despite the similar intrinsic iron content between human (0.59 mg/L) and bovine milk (0.69 mg/L), the iron bioavailability of human milk (49%) is significantly greater than that of cow's milk (19%) (Saarinen et al., 1977). This lower bioavailability of iron in cow's milk has mainly been attributed to high calcium and protein contents, especially the casein contained in this food (Hurrell et al., 1988). The enhancing effect of human milk on iron absorption used to be accredited to lactoferrin, ascorbic acid, lactose and cysteine that are greater in concentration as compared to bovine milk. However, lactoferrin, an Fe-binding protein and the major enhancing factor from human milk, has been consistently shown to have a limited or even a negative effect to improve iron absorption in the adult intestinal tract.

The researches then have been focused on the effects of bioactive peptides from enzymatically hydrolyzed milk proteins on iron absorption (García-Nebot et al., 2010; Kibangou et al., 2005; Argyri et al., 2007; Caetano-Silva et al., 2015). Upon hydrolysis, milk proteins yield certain low molecular weight peptides that may exert positive effects on iron absorption. For instance, low molecular weight caseinophosphopeptide (CPP) produced from casein has a positive effect on iron absorption (García-Nebot et al., 2010; Kibangou et al., 2005). The low molecular weight peptides from hydrolyzed whey may also improve the iron solubility and further enhance its uptake by intestinal epithelial (Caco-2) cells (Caetano-Silva et al., 2015). However, the enhancing effect of hydrolyzed milk peptides on iron absorption tends to be masked in the whole milk digest. To date, the Fe uptake– enhancing component in human milk, the so-called milk factor, has not been found.

2.6.4. Strategies to address iron deficiency anemia

Iron deficiency anaemia (IDA) accounts for 50% of all anaemias and affects 1/4 of the world population include with infants, children and pre-menopausal women at most risk (Mclean, Gogswell, Egli, and Benoist, 2008). Both in developing and developed countries the consequences

of anaemia result in significant health problems and economic cost. Nutritional iron deficiency is a state of imbalance when the iron content of the diet is insufficient to meet physiological needs (Aggett et al., 2010).

Supplementation of food with different iron salts is the best way to address nutrient deficiency in populations. Several studies have investigated the benefits of iron supplementation to improve iron status and alleviation of the adverse consequences of iron deficiency and anemia. Many studies have reported reduced risk of IDA in children and pregnant women due to iron supplementation (Harrison and Arosio, 1996; McKie, 2008). Sources of elemental iron for iron supplementation include ferrous sulfate, ferrous fumarate or ferrous gluconate (Vargas, 2003). Recently, there have been concerns about the safety of iron supplementation due to the chance of excess iron or iron overload, which could act as transition metal ions causing oxidative damage if they are not bound to any iron storage or transport proteins.

Fortification is another strategy that is used to help to mitigate IDA. The effect of iron fortified foods on iron status in different populations has been widely studied. The studies have used chelating vehicles such as EDTA and citric acid or those with reducing power such as ascorbic acid (AA) fortified with iron to improve iron status of subjects (Hurrell et al., 2010). Troesch et al., (2011) also measured the serum non-transferrin-bound iron (NTBI) in women after giving oral AA-FeSO4 and NaFeEDTA. The results showed that iron fortifications used in the study do not increase NTBI in women, suggesting a low risk for adverse health consequences as described in the previous method. Iron fortification is not as fast as supplementation in addressing nutrient deficiency, but it is an effective approach since its impact is much more sustainable in the long-term. It is also important that the vehicles used do not alter the organoleptic properties of the food and are cost-effective (Hallberg et al., 1989).

Apart from supplementation and food fortification, one effective but often neglected strategy for alleviating iron deficiency is dietary modification/intervention to increase the intake of dietary components that promote iron absorption from low-bioavailability meals (Heath et al., 2001). While iron supplementation represents a short-to-medium term approach to address iron deficiency and anemia, food fortification and dietary diversification represent medium-to-long term approaches (Ahluwalia, 2002). Patterson et al. (2001) compared the effect of a dietary intervention with iron supplementation in improving iron status among iron-deficient women. They found that although simple iron supplementation improved ferritin levels faster than the diet treated group, the ferritin level in the diet group continued to increase during the 6 months follow up period. Typically, increasing overall food intake is associated with an increased intake of iron as well as other nutrients. The bioavailability of iron from the food is the utmost important in addressing iron deficiency. To ensure iron bioavailability for absorption, animal source foods, as well as plant-based iron consumed together with fruits and vegetables rich in vitamin C must be regular components of the diet. Dietary inhibitors of iron absorption must not be consumed together with the iron containing meal, especially for plant-based diets. Strategies to reduce the effect of these inhibitors include soaking or fermenting grains to minimize the phytate content and avoiding the ingestion of calcium supplements or dairy products high in calcium together with the iron containing meal (Thompson, 2011).

2.6.5. Methods of assessing nonheme iron bioavailability

Several methods have been used to assess non-heme iron bioavailability. Forbes et al. (1989) reviewed some of these methods and discussed their validity to human iron absorption. Rat models are applicable for *in vivo* studies, and there have been models using laboratory anemic rat/mice for studying non-heme iron absorption. *In vitro* studies are more applicable in the early stage of a

study due to their lower cost, decreased complexity, and they are not time consuming. The equilibrium dialysis method is the most common method used to assess iron bioavailability (Miller et al. 1981; Shen et al. 1994). Bioavailability based on solubility or dialyzability alone has been shown to not accurately reflect uptake by the intestinal mucosa (Gangloff et al., 1996; Miller and Bemer., 1989). Since foods are comprised of many different compounds, a non-heme iron absorption model that allows one to test many combinations of substances and individual compounds under more physiological conditions facilitates the identification of factors responsible for enhancing non-heme iron absorption.

The Caco-2 cell line appears to be a good model to measure physiological iron absorption (Garcia et al. 1996, Glahn et al. 1996, Au and Reddy 2000). The Caco-2 cell is a human colon carcinoma cell, which undergoes spontaneous differentiation in cell culture to form polarized epithelial cell monolayers with many of the characteristics of enterocytes (Pinto et al. 1983). Caco-2 cells combined with *in vitro* digestion of the test component or meal have been developed by Glahn et al. (1998) and was shown to be useful in measuring non-heme iron absorption under a variety of conditions (Figure 2.8). Many factors that influence Caco-2 cell growth and non-heme iron uptake have been studied (Han. et al. 1995; Arredondo et al. 1997; Alverez-Hemandez et al. 1998). Use of the Caco-2 cell model has been shown to be a valid tool to study human non-heme iron bioavailability, including the enhancing effect of possible meat and milk factor candidates on non-heme iron absorption (Garcia et al. 1996; Glahn et al. 1996; Au and Reddy, 2000). In addition, iron absorption by Caco-2 cells correlates well to human iron absorption measurements (Au and Reddy, 2000). Use of the Caco-2 cell model allows one to determine the interaction among various food components individually and collectively. In addition, use of the Caco-2 cell model to study iron absorption is relatively inexpensive when compared to human studies or animal models.

Studies showing the validity of using the Caco-2 cell model to study human iron bioavailability, including the enhancement of meat on non-heme iron absorption (García-Nebot et al., 2010; Kibangou et al., 2005; Agryri et al., 2007; Caetano-Silva et al., 2015), prompted us to use this cell line to identify, and thereafter characterize, beef proteins that enhance non-heme iron bioavailability.

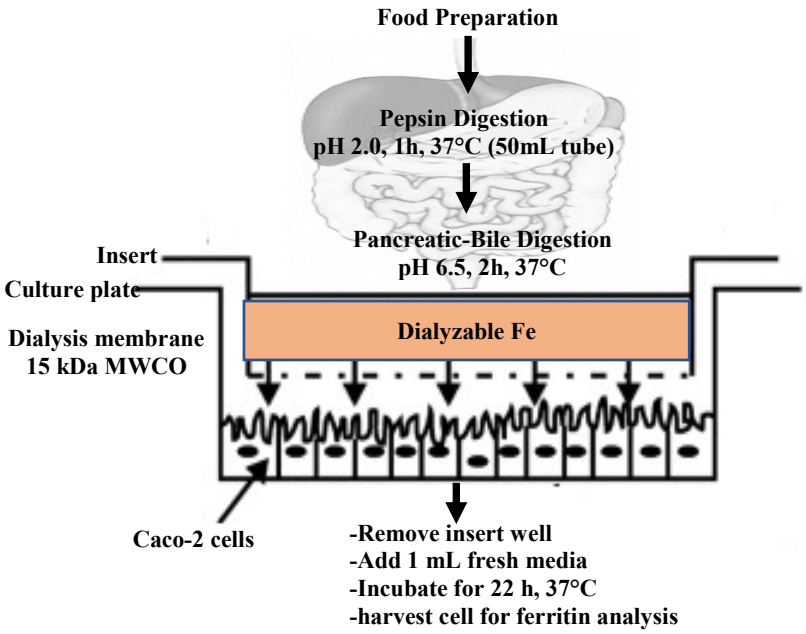

Figure 2. 8. Schematic diagram of *in vitro* digestion, a Caco-2 cell model adopted from Glahn et al (1998) with permission from Oxford university press.

2.6.6. The effect of sulfated GAGs on nonheme iron absorption

As discussed in the meat factor section, the enhancement of the meat protein and their constituents on non-heme iron are suggested to be involved in many different mechanisms including both chelating and solubilization. Recently, GAGs, as the only carbohydrate constituent from the ECM of muscle tissues in the form of PGs, are proposed to also play a part in the meat factor. The first study with proof of the involvement of GAGs in the non-heme iron absorption was reported by Huh et al. (2004). The study was aimed to evaluate the effect of cooked fish

haddock fillet on non-heme iron absorption by the *in vitro* Caco-2 cell model. The acid (pH 2.0) extract from the lyophilized cooked fish was fractionated by Sephadex G-25 size exclusion column and showed increased Caco-2 iron uptake approximately by 9-fold. However, when these fractions were further purified by C18 reverse-phase HPLC, the enhancing activity was observed only in the lower molecular weight fraction (< 5 kDa). The combined content analysis showed that the active fraction contains high amounts of carbohydrates and negligible amounts of proteins and amino acids. The authors suggested that these low molecular weight carbohydrates could be the oligosaccharides generated from GAGs in the fish muscle tissue during the acidic treatment. Later, Hurrell et al. (2006) suggested that the meat factor attribute by meat protein/peptides is indispensable but also that it is unlikely for a single factor to be involved. The author did not rule out the possibility of the involvement of non-protein enhancer for Fe absorption in the chicken protein isolates and suggested that GAGs could be a potential candidate. In a follow up study based on the discovery from Huh et al (2004), by isolating GAGs from cooked fish muscle meat and applying it alone with $FeCl_3$ in a simulated gastrointestinal digestion exposed to Caco-2 cells, the positive effect that GAGs contribute to the meat factor was comfirmed. More importantly, the presence of CS/DS-related structures were identified in the isolated GAGs from cooked haddock, which provide some speculation regarding the mechanism for its enhancing effect on non-heme iron (Fe^{3+}). As discussed previously in section 2.5, CS showed superior antioxidant property as compared to the other GAGs due to their unique chemical structure. The antioxidant activity of CS had been proven through two possible pathways:1) by directly scavenging ROS, 2) by chelating transition metals such as Fe^{2+}. Also, due to the negative charge that the CS structure carried, it may also be capable of transferring one electron thus to reduce the Fe^{3+} to Fe^{2+}, which is then more bioavailable for intestinal absorption. Interestingly, this was also suggested as the mechanism for

cysteine's promotion effect on non-heme iron absorption (Kapsokefalou and Miller, 1991). Thus, it is possible that the antioxidant mechanisms of CS may relate to its enhancing effect on non-heme iron absorption as illustrated in Figure 2.9. The dietary ferric ion is usually present as insoluble and it is one of the major causes of its poor bioavailability. The ferrous ion on the other hand is very reactive when it is free of bonding and could be easily oxidized to ferric form through the Haber-Weiss reaction (Figure 2.5). The ferric ion could react with water to form insoluble $Fe(OH)_3$ leading to the precipitation of the iron. The intervention of antioxidant GAGs or more specific CS in all the reactions described above may maintain a sufficient amount of dietary iron in its soluble form by forming a soluble iron-CS complex or by preventing the soluble iron (Fe^{2+}) from oxidized (Fe^{3+}) due to its scavenging and reducing capacities (Figure 2.9). The *in vitro* digestion still could be the primary factor that is responsible for initiating the enhancing effect of GAGs. By not only releasing the bonded sulfate GAGs from its protein core (proteoglycan), the digestion process would create a lower acid environment that may hydrolyze the GAG polysaccharides and produce some smaller GAG-derived oligosaccharides, which then may be more effectively absorbed by the enterocytes.

Furthermore, the effect of GAG on iron had a direct influence in cell biology preserving the cellular redox status (Laparra et al., 2009). There remains a concern since an increase in soluble iron content could increase free radical production in the colon to a level that could cause mucosal cell damage (Lund et al., 1999). To resolve this concern, another study on the effect of GAGs on non-heme iron uptake by Caco-2 cell models was carried out with the aim to better understand their enhancing mechanism. This study employed biomarker monitoring of cellular metabolism during the exposure of GAG/Fe (Laparra et al., 2009). All Fe-containing solutions tested caused a sharp intramitochondrial accumulation of ROS. However, cell cultures exposed to the GAG/

Fe^{+3} mixture exhibited a more preserved (by 8%) intracellular GSH concentration compared to cultures exposed to Fe^{+3} or Fe^{+3}/AA mixture. This suggested that the GAGs exerted an antioxidant effect on the colonic mucosal cells when exposed to iron source, since the protection effect of CS on some enzymatic antioxidants (i.e. extracellular superoxide dismutase) is well known (Karlsson et al. 1988). The study also proposed that Fe could be internalized into cells by endocytosis through the mediation of GAGs on the membrane of the enterocyte in addition to the DcytB and DMT1 pathways.

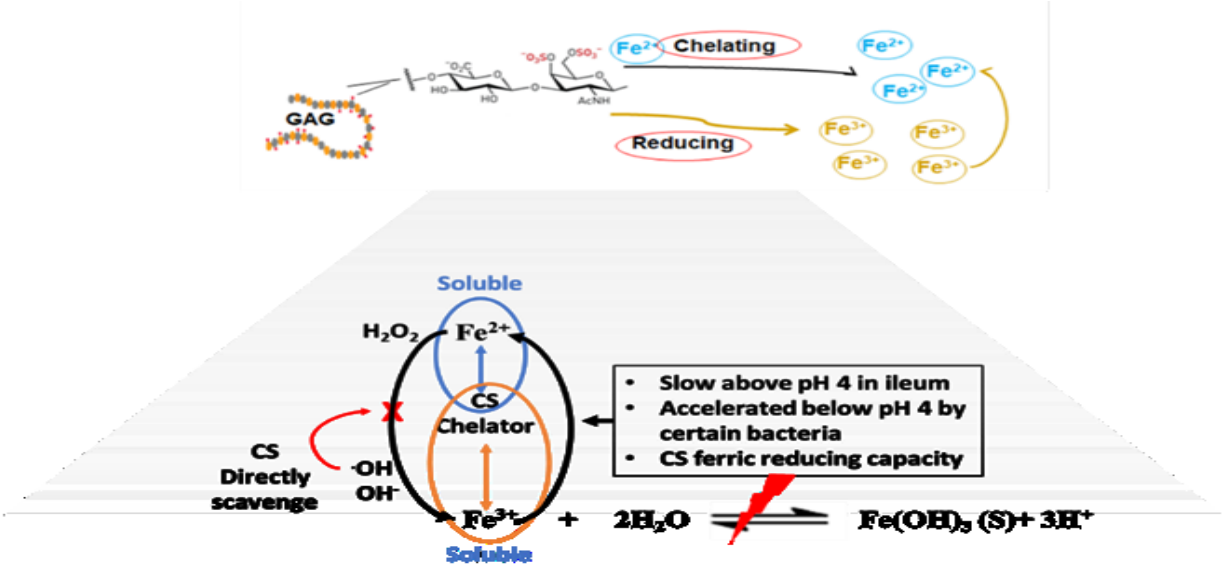

Figure 2. 9. The interactions between GAGs (CS) and iron.

The accumulated results from all these studies suggest that GAGs likely play an important role in enhancing non-heme iron absorption. Their enhancing effects on iron absorption and whether the mechanisms involved relate to their antioxidant properties are still poorly understood. A greater knowledge of these effects and mechanisms would be useful for designing diets with enhanced iron bioavailability, as well as for developing food products that appeal to health-conscious consumers.

Chapter 3. Sulfated glycosaminoglycan-derived oligosaccharides produced from chicken connective tissue promote iron uptake in a human intestinal Caco-2 cell line[1]

3.1. Introduction

The broiler chicken meat processing industry produces a large number 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 et al., 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 the 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 glycans bound to the protein core; and on the other hand, hyaluronic acid (HA), the non-sulfated GAG polysaccharides, 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, and Yamamoto, 1989). HS and DS also have anticoagulant activity (Volpi, 2006). Besides these known properties, the *in vitro* studies suggest that GAG polysaccharides can enhance non-heme iron absorption, thereby improving one's nutritional iron status (Huh, Hotchkiss, Brouillette, and

[1] *A version of this chapter has been published in Food Chemistry. (Wang H., and Betti M. 2017. Food Chemistry. 220:460-469.).*

Glahn, 2004; Laparra, Tako, Glahn, and Miller, 2008; Laparra, Barbera, Alegria, Glahn, and Miller, 2009). 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, and 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) had demonstrated that the ferritin synthesis in Caco-2 cells combined with the *in vitro* gastrointestinal digestion is a good model for studying the iron bioavailability. However, the mechanisms regarding sulfated GAG 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, Frohman, Thannhauser, Welch and Glahn, 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 and Xie, 2015). Besides this particular reducing capacity, Laparra et al. (2009) also suggested that Fe could also 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, Horler, Moser, Hofer, Fehr and Wagenhauser, 1992). Several studies reported in recent years have demonstrated that reducing the CS molecular weight and even further creating an oral delivery system with amphiphilic polysaccharides, liposomes and addition of absorption enhancers, could be possible ways 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 uptake of iron through 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 my 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.

3.2. Materials and methods

3.2.1. 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, and 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 haparinum,* 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).

3.2.3. 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 cut off (MWCO) was performed. The digests were precipitated with 70%

ethanol. The total GAGs precipitate was dissolved in deionized water and then fractionated by using anion exchange chromatography with sequential elution with 0.4 M and 2.0 M NaCl. The sulfated GAG polysaccharides were produced from chicken skin and cartilage for later experiments. DPPH scavenging activities, ferric reducing activities and iron chelating activities were determined to estimate the antioxidant capacities of the isolated sulfated GAG polysaccharides and their enzymatically depolymerized oligosaccharides. The experiment consisted of a total of 4 treatments applied to three independent and isolated batches. In the second part of the study, the effect of test samples on Fe uptake by the Caco-2 cell culture modeled after simulated gastrointestinal digestion was investigated. Test samples included sulfated GAG polysaccharides and enzymatically depolymerized sulfated GAG oligosaccharides from chicken skin and cartilage. Three independent cell trials were conducted and each treatment was performed in triplicate.

3.2.3. 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) (Figure 3.1). Chicken skin was cut into small pieces and washed with distilled water (1:5, w/v) then stirred for 20 min to remove excess blood and some impurities. After that hey 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. For the cartilage, they were washed with distilled water to remove blood and the adhering muscle tissues. The chicken skin and cartilage were digested with pancreatic enzymes. Tissue samples were first boiled in water

(1:5, w/v) for 10 min, cooled to room temperature (24 °C) and then homogenized. Pancreatin was added to the homogenate at 1% sample weight, and the pH was adjusted to 7.6.

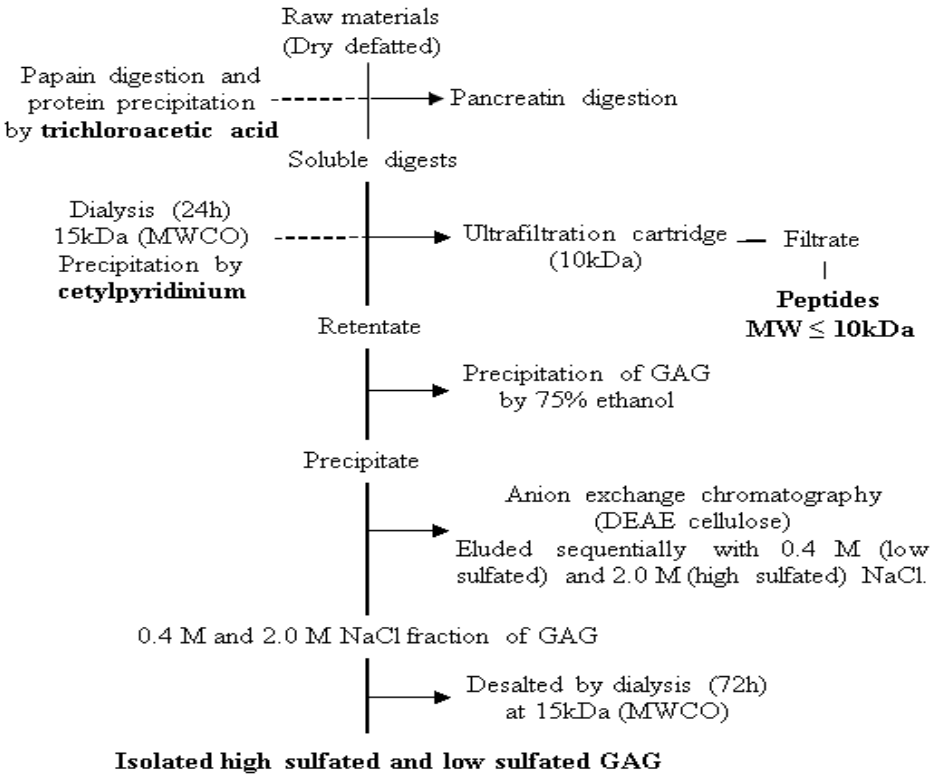

Figure 3. 1. Scheme for food-grade glycosaminoglycans extraction. - - - indicate the chemicals that involved in the nonfood-grade glycosaminoglycans extraction. (Nakano et al., 2012)

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 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 an ultrafiltration system (Minimate^{TM} TFF system, Pall Corporation, MI, USA) using a capsule with a 10 kDa (MWCO) to remove soluble peptides (< 10 kDa) generated during the proteolysis. Three volumes of absolute ethanol were added to completely precipitate the GAGs.

The total precipitated GAGs were then collected by centrifugation at 27,200 × *g,* 4°C for 20 min.

The total GAG polysaccharides were then dissolved in deionized water and applied to an anion exchange column (1.5 cm × 5 cm) of DEAE cellulose, which was equilibrated with water and adjusted to pH 3.0 with 2.0 M HCl. The column-bound GAG polysaccharides were eluted stepwise first with 0.4 M NaCl (pH 3.0), and then 2.0 M NaCl (pH 3.0) was applied (the GAG elution chromatograph was previously determined with a linear NaCl gradient of increasing molarity, data not shown). The flow rate of the column was 1.7 mL/min. The carbazole reaction was used to monitor GAG uronic acid. Two fractions (the 0.4 and 2.0 M) were thus obtained from both cartilage and skin tissues. It was expected that the sulfated GAG fraction (mainly rich in CS or DS) was eluted with 2.0 M NaCl and that the less or "non-sulfated" one – mainly rich in HA – was eluted with the 0.4 M NaCl. The fractions were dialyzed in deionized water using 15 kDa MWCO dialysis tube to remove salts, and then lyophilized for further analysis. This experiment was carried out in triplicate.

3.2.4. Uronic acid, sulfate and protein content analysis

The GAG content was estimated by determining the uronic acid content in the sample according to Nakano, Sunwoo, Li, Price and Sim. (1996), which was carried out by the carbazole reaction (Kosakai and Yoshizawa, 1979). The sulfate content in the GAGs was determined with the sodium rhodizohate method according to Terho and Hartiala (1970). The protein content was determined using the Bradford assay (Bradford, 1976) with bovine serum albumin as a standard.

3.2.5. Separation and fractionation of sulfated GAG polysaccharides

The sulfated GAG polysaccharides from skin and cartilage were further fractionated by selective precipitation using varying final concentrations of ethanol according to Nakano et al.

(2012). Five fractions (I-V) were collected at ethanol concentrations of 18%, 25%, 40%, 50% and 73%, respectively.

3.2.6. Cellulose acetate electrophoresis

Fractionated sulfated GAG polysaccharides were digested with chondroitinase-ABC and chondroitinase-ACII with 5.0 µg uronic acid/0.03 unit of enzyme and 1.0 mg uronic acid/0.005 unit of enzyme, respectively, in 0.01 M sodium acetate buffer containing 0.02% sodium azide at 37°C for 1 h. The pH of the buffer was 8.0 for chondroitinase-ABC and 6.0 for chondroitinase-ACII (Nakano et al., 1996).

Electrophoresis on cellulose acetate strips was carried out for all samples (1.0 mg/mL) before and after the chondroitinase digestions in 0.1 M pyridine/0.47 M formic acid at pH 3.0 according to Hata and Nagai (1971). Fractionated skin sulfated GAGs precipitated with 40% and 50% ethanol and fractionated cartilage sulfated GAGs precipitated with 40% ethanol with and without enzymatic digestion were examined (see section 3.2.5). After electrophoresis, each strip was stained in 0.1% (w/v) Alcian blue 8GX in 0.1% acetic acid and washed extensively with deionized water.

3.2.7. Production of sulfated GAG oligosaccharides (enzymatic depolymerisation)

Digestion with testicular hyaluronidase was carried out by incubating the sulfated GAG polysaccharide samples from chicken skin and cartilage each containing 30 µg of uronic acid with 40 units of enzyme in 1.0 mL of 0.04 M sodium phosphate buffer containing 0.02 M citric acid at pH 5.0 and 37°C for 17 h (Suzuki, 1969). The cellulose acetate electrophoresis was used to determine the completion of the digestion (Nakano et al., 2012). The desalting step was done by

Rexyn I-300 (H-OH) desalting beads. The freeze dried sulfated GAG polysaccharides and depolymerized oligosaccharides were collected and stored at -20°C for further analysis.

3.2.8. Determination of antioxidant capacity

3.2.8.1. 2, 2-Diphenyl-1-picrylhydazyl (DPPH) radical scavenging activity

The DPPH radical scavenging effect was assessed by the discoloration of a methanol solution of DPPH. This assay is based on the method developed by Blois (1984). The DPPH solution turns from a deep violet color to yellow when it reacts with a reducing agent. For this experiment, a microplate assay method was used according to Li et al. (2012) since it is rapid, sample-saving and environmentally friendly. DPPH was dissolved in absolute methanol to 200 µM and prepared daily. A 100 µL aliquot of sample was added to 100 µL DPPH solution with final concentration of 1.0 mg/mL into a 96-well microplate. The plate was shaken vigorously for 2 min and the absorbance of the mixtures was measured with a microplate reader (SpectraMax M3, Molecular Devices, Sunnyvale, CA, USA) at 517 nm after 60 min incubation in darkness at 37°C. Ascorbic acid was used as an antioxidant standard and positive control. DPPH radical scavenging activity was expressed as the percentage inhibition and was calculated using the following formula:

DPPH radical scavenging activity (%) = $[(Abs_{control} - Abs_{sample}) / (Abs_{control})] \times 100$

$Abs_{control:}$ absorbance of DPPH radical + methanol

$Abs_{sample:}$ absorbance DPPH radical + sample or ascorbic acid.

3.2.8.2. Ferric reducing activity

The ferric reductase assay was performed as described previously with modifications (Timmerman and Woods, 1999). Briefly, the reaction mixture containing the sample, ferric chloride (50 µM) and ferrozine (5.0 mM) was adjusted to a total volume of 0.2 mL with a final

sample concentration of 1.0 mg/mL and distributed into a microplate. The plate was shaken vigorously and incubated at room temperature (24 °C) for 30 min. Ascorbic acid was used as an antioxidant standard and positive control. The absorbencies of the mixtures were measured at 562 nm using a Spectramax 3 microplate reader. Formation of the ferrous iron was calculated by using a standard curve prepared with ammonium ferrous sulfate and the specific activity expressed as ferrous iron formed (μM)/ferric iron added (μM).

3.2.8.3. Iron chelating activity

The ferrous iron-chelating potential of the samples was determined according to the method described by Decker and Welch (1990) with minor modifications. The reaction mixture, containing the sample, ferrous chloride (0.5 mM) and ferrozine (5.0 mM), was adjusted to a total volume of 0.2 mL with final sample concertation of 1.0 mg/mL. The plate was shaken vigorously and incubated at room temperature (24 °C) for 30 min. The absorbance of the mixture was measured at 562 nm against a blank. The blank was prepared in the same manner except that deionized water was used instead of sample. Ethylenediaminetetra-acetic acid (EDTA) was used as a positive control. The percentage inhibition of ferrozine-Fe^{2+}complex was calculated using the following formula:

Iron-chelating activity (%) = $[(Abs_{control} - Abs_{sample}) / Abs_{control}] \times 100$

$Abs_{control:}$ absorbance of the blank

$Abs_{sample:}$ absorbance of the sample or EDTA

Commercial laboratory grade CS from bovine trachea and DS from porcine intestinal mucosa were used as standard references.

3.2.9. Cell culture

Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD., USA) and used in all experiments described in this study between passages 33-38. Cells were routinely cultured at 37°C in an incubator with a 5% CO_2, 95% air atmosphere at constant humidity in Dulbecco's Modified Eagle Medium (DMEM, Gibco) under conditions described by Glahn et al. (1998).

The cells were used for Fe uptake experiments at 21 d post seeding. For the assays, Caco-2 cells were seeded at 50,000 cells/cm^2 in 12-well Transwell permeable support plates with pore size 0.4 µm (Costar®, Corning Inc., NY, USA) (Eady, Wormstone, Heaton, Hilhorst and Elliott, 2015). The medium was refreshed every 2-3 d. Trans-epithelial electrical resistance (TEER) was utilized, using an EVOM2 Epithelial Voltohmmeter (World Precision Instruments, Hitchin, UK), over subsequent days to confirm the time to confluence and the permeability of the cell monolayers. The MTT (3-[4,5-dimethylthiazol-2-yl]-2,3-diphenyl tetrazolium bromide) cell proliferation assays (American Type Culture Collection, Manassas, VA, USA) were performed with all the samples at a range of concentrations (0 to 2.0 mg/mL) with 0.1 mg/mL interval (data not shown). On the day prior to the *in vitro* digestion experiment, the DMEM was removed and washed three time with 1.0 mL of minimal essential medium (MEM, Gibco). Then, 1.0 mL MEM was added to the cells and returned to the incubator.

3.2.10. In vitro digestion

The human gastrointestinal digestion process was stimulated using the *in vitro* approach developed by Glahn et al. (1998). Aliquots of CS from bovine trachea, DS from porcine intestinal mucosa, chicken cartilage and skin sulfated polysaccharides with and without enzymatic depolymerisation were subjected to *in vitro* digestion. All samples were mixed with 50 µmol/L

$FeCl_3$ at final concentration of 0.8 mg/mL (based on the MTT cell proliferation assay). A digest containing $FeCl_3$ (50 μmol/L)/ ascorbic acid (AA) mixture (molar ratio for Fe/AA of 1/20) was used as the positive control. The baseline cell ferritin was measured as blank. Peptic and intestinal digestions were conducted on a rocking platform shaker and placed in an incubator (37°C/5% CO_2/95% relative humidity). After the gastric step (pepsin/pH 2.0/1 h), a 1.0 mL aliquot of gastrointestinal digest (final sample concentration of 0.8 mg/mL) was loaded into the apical chamber of the two-chamber system in 12-well permeable support plates and incubated for 2 h at 37°C. MEM (1.0 mL) was added to the basolateral chamber. After 2 h, the apical and basolateral media were removed and collected into separate tubes. The apical surface of the cells was washed twice with 1.0 mL of PBS (pH 7.4) (10 mM phosphate buffer, 2.7 mM KCl, 137 mM NaCl) and an additional 1.0 mL of MEM were added. The cells were incubated for 22 h at 37°C.

3.2.11. Ferritin Analysis

Exactly 24 h after the start of the Fe uptake period, the MEM medium was removed from the apical chamber and the apical surface of the cells was washed twice with 1.0 mL PBS. The ferritin concentration and total protein concentration were determined on aliquots of the harvested cell suspension with 1-stage sandwich immunoradiometric assay (Feriron II Ferritin Assay, Ramco laboratories, Houston, TX, USA) and BCA protein assay kit (Pierce™ , Fisher Scientific, Toronto, ON, Canada), respectively. Ferritin formation was used as an index of the cellular Fe uptake. Therefore, the ratio of ferritin/total cell protein expressed as ng of ferritin per mg of protein was used to estimate the content of ferritin formed in Caco-2 cells.

3.2.12. Statistical analysis

The extraction experiments were repeated three times. All bioassay results were expressed as means ± standard deviation (SD). The experimental data were subjected to the tow-way analysis

of variance (ANOVA) and the significance of the differences between the treatments was determined using theTukey test with $p < 0.05$.

3.3. Results and discussion

3.3.1. Extraction and isolation of sulfated GAG polysaccharides from chicken tissues

Different selective extraction procedures and precipitation techniques have been used to isolate and obtain different types of sulfated GAG polysaccharides. Various attempts have been made to minimize the use of organic toxic solvents and chemicals during the extraction and separation processes (Nakano et al., 2012; Srichamroen, Nakano, Pietrasik, Ozimek and Betti, 2013). A food-grade extraction according to the methodology of Nakano et al. (2012) was conducted on pre-cleaned chicken skin and cartilage tissues. This extraction involved the use of an economical proteinase for liberating the sulfated GAG polysaccharides from the extracellular matrix and a subsequent membrane ultrafiltration at 10 kDa MWCO to replace the common hazardous chemical deproteinization step involving trichloroacetic acid. After ultrafiltration, 0.4 M and 2.0 M NaCl fractions were collected by anion exchange chromatography. The two fractions were then subjected to uronic acid, sulfate and peptides analyses (section 3.2.4) in order to verify their quality (Table 3.1). The 2.0 M NaCl fractions contained significantly greater amounts of uronic acid and sulfate compared to the 0.4 M NaCl fractions ($p < 0.05$). This indicates that the majority of sulfated GAG polysaccharides from chicken skin and cartilage were eluted with 2.0 M NaCl. With regard to the peptide content (Appendix A and B), Nakano et al. (2012) reported a 0.1 peptide to uronic acid ratio in the sulfated GAGs from chicken biomass by pancreatin digestion. In this study, the peptide to uronic acid ratio in the 2.0 M NaCl fraction of cartilage and skin were apparently much less with a weight ratio below 0.05. This suggests that for this food grade GAGs extraction, pancreatin digestion effectively liberates the covalently attached sulfated GAGs from the protein

core with minimal peptide chain attached. Several studies have postulated that the protein or peptide moiety in polysaccharides is responsible for part of their antioxidant properties

(Leung, Zhao, Ho, and Wu, 2009; Liu, Sheng, and Li, 2013). Therefore, it was critical in this study to obtain sulfated GAG polysaccharides with minimal peptide contamination to correctly assess the bioactivity of these sulfated polysaccharides.

Table 3. 1. Analysis of the extracted GAGs from chicken skin and cartilage.

	0.4 M NaCl fraction		2.0 M NaCl fraction	
	Cartilage	Skin	Cartilage	Skin
Uronic acid (μg/mg)	73 ± 4^{c}	81 ± 6^{c}	258 ± 6^{a}	193 ± 17^{b}
Sulfate (μg/mg)	29 ± 1^{c}	17 ± 2^{d}	181 ± 5^{a}	134 ± 12^{b}

Given values are expressed as μg/mg dry weight for GAGs. Means in the same row with different letters are significantly ($p<0.05$) different according to the Turkey test.

To verify the identity of GAG polysaccharides in the 0.4 and 2.0 M NaCl fractions, cellulose acetate electrophoresis was performed. This test is based on the charge density of the GAGs - the ones with more negatively charged groups will move faster and end up with a higher mobility than the one with less or none. The mobilities of GAG polysaccharides from 0.4 M and 2.0 M NaCl fractions were compared to the mobilities of standard CS, DS and HA. The CS standard has the greatest amount of negatively charged sulfate groups indicated by the band on the top of the membrane (lane 1) followed by the DS standard (lane 2) (Figure 3. 2). This slight difference of band position is due to the difference in the structures of CS and DS, where CS is epimerized to form DS. HA, the non-sulfated GAG was located at the bottom of the membrane (lane 3). The sulfated GAG polysaccharides from cartilage (lane 4) had an intensive band with its mobility identical to that of standard CS. This suggests that the majority of the GAG polysaccharides in the cartilage were CS. While the sulfated polysaccharides extracted from the skin showed a broader band (Lane 6) as compared to those extracted from the cartilage sample, the average mobility was

between those of the CS and DS standard. This pattern implies the possibility that the majority of the sulfated polysaccharide from the skin are constituted by CS/DS copolymers. This CS/DS structure has been reported as the major GAG structure found in the sulfate containing fraction of the haddock (Laparra et al., 2008) and atlantic cod (Tingbø, Kolset, Ofstad, Enersen and Hannesson, 2005). On the other hand, 0.4 M fractions of cartilage (lane 5) and skin (lane 7) both had one weakly stained band that migrated as far as the HA standard. This indicates that HA (non-sulfated GAG) was efficiently separated from the sulfated fraction. Since the 2.0 M NaCl fractions from skin and cartilage tissues contain the majority of sulfated GAG polysaccharides, they were used for further analysis in order to verify the types of GAG present.

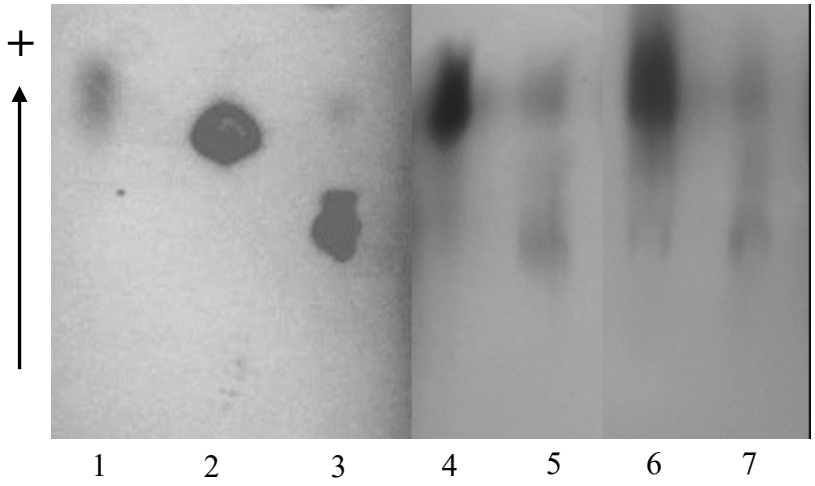

Figure 3. 2. Cellulose acetate electrophoresis of the 2.0 M and 0.4 M GAG fractions extracted from different broiler chicken tissues. Lane 1: CS standard; Lane 2: DS standard; Lane 3: HA standard; Lane 4: Cartilage 2 M GAGs; Lane 5: Cartilage 0.4 M GAGs; Lane 6: Skin 2 M GAGs; Lane 7: Skin 0.4 M GAGs. The arrow indicates the direction of mobility.

3.3.2. Fractionation of sulfated GAG polysaccharides by differential precipitation with ethanol

The sulfated GAG polysaccharides obtained from chicken cartilage and skin obtained by anion exchange chromatography with 2.0 M NaCl were selectively fractionated with different concentrations of ethanol (Table 3.2). For the cartilage sample, the majority (> 75%) of the total uronic acid was recovered in fraction III (obtained by precipitation with 40% ethanol). This is

consistent with the report of Nakano et al. (2012) who studied chicken biomass GAGs and found that most (average 81%) of the total uronic acid of sulfated GAGs were recovered by precipitation with 40% ethanol. For the skin sample, the largest amount of uronic acid was recovered in fraction III and IV.

Table 3. 2. Fractionation of sulfated GAGs (2 M NaCl fraction) from chicken cartilage and skin by differential precipitation with ethanol

Material	Fraction*	% of total uronic acid
Cartilage sulfated GAGs	I	0.3
	II	1.4
	III	75.6
	IV	16.5
	V	5.2
Skin sulfated GAGs	I	9.7
	II	16.7
	III	42.6
	IV	23.7
	V	8.3

*I, II, III, IV and V represent sulfated GAG (2.0 M NaCl) fractions obtained by precipitation with 18%, 25%, 40%, 50% and 73% ethanol, respectively.

The recovered ethanol fractions (cartilage fraction III, skin fractions III and IV) were subjected to selective digestion with chondroitinase AC, and chondrotinase ABC, and then cellulose acetate electrophoresis was conducted to identify the types of sulfated GAG polysaccharides represented in these fractions. The minor fractions, however, were not characterized further due to the limited amount of sample. Chondroitinase AC only digested CS structures while chondroitinase ABC cleaved both CS and DS. The standard CS, skin fraction IV and cartilage fraction III were susceptible to both chondroitinase AC and ABC (Figure 3.3A, B: lanes 2, 8 and 10, respectively) which implies that the most abundant sulfated GAG polysaccharide in this fraction was CS. On the other hand, the standard DS and skin fraction III were not susceptible to chondroitinase AC

digestion but were susceptible to chondroitinase ABC (Figure 3.3A, B: lanes 4 and 6, respectively), suggesting that most of the sulfated GAG from skin is DS. In summary, CS is the only polysaccharide found in the sulfated GAG from the chicken cartilage, while a mixture of CS and DS, the so-called CS/DS copolymer, is the major polysaccharide present in the skin. This is consistent with the study of Nandini, Itoh and Sugahara, (2005), in which the authors showed that the extracted GAG from the skin of blue shark (*Prionace glauca*) was composed of CS/DS hybrid chains with a unique heterogeneous sulfation pattern. Nakano et al. (2012) have also observed a similar electrophoresis pattern for the sulfated GAG polysaccharides that were extracted from chicken skin.

3.3.3. Production of sulfated GAG oligosaccharides (enzymatic depolymerisation) from the 2.0 M NaCl fraction

The depolymerisation of the sulfated GAG polysaccharides from chicken cartilage and skin has been achieved by enzymatic hydrolysis using bovine testicular hyaluronidase. It is an endo-β-*N*-acetyl-D-hexosaminidase that hydrolyzes HA and CS at the β-1, 4-*N*-acetylglucosamine bonds endolytically producing a mixture of disaccharide, tetra-saccharide and oligosaccharide units (Honda, Kaneiwa, Mizumoto, Sugahara and Yamada, 2002). Fransson and Roden (1967) also investigated the products obtained after digestion of DS with testicular hyaluronidase and reported a number of oligosaccharides ranging in molecular weight from 500 to 16,000 Da. As shown in Figure 3.3C, results with testicular hyaluronidase digestion were consistent with those with

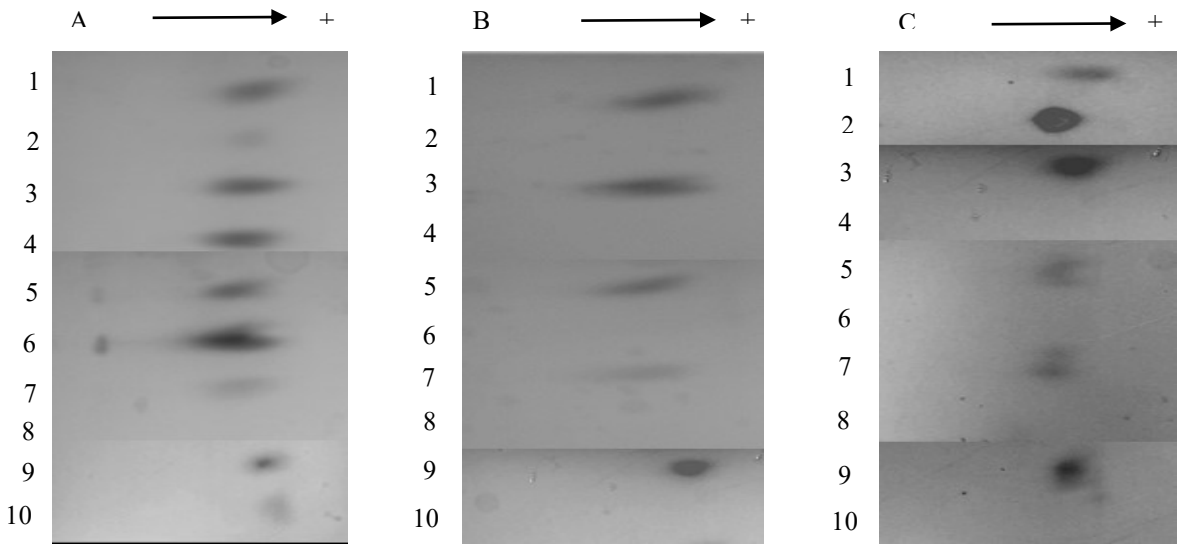

Figure 3. 3. Electrophoresis of the fraction III from cartilage sulfated GAGs and fraction III and IV from skin sulfated GAGs before and after enzymatic digestion. (A) Digested by Chondroitinase AC, Lane 1, 3, 5, 7, 9 are CS standard, DS standard, Skin III, Skin IV and Cartilage III without enzyme; Lane 2, 4, 6, 8, 10 are with enzyme. (B) Digested by chondroitinase ABC, Lane 1, 3, 5, 7, 9 are CS standard, DS standard, Skin III, Skin IV and Cartilage III without enzyme; Lane 2, 4, 6, 8, 10 are with enzyme. (C) Digested by hyaluronidase, Lane 1 and 2 are CS and DS standard; Lane 3, 5, 7, 9 are CS standard, DS standard, Skin and Cartilage sulfated GAGs without enzyme; Lane 4, 6, 8, 10 are with enzyme. The arrow indicates the direction of the mobility.

chondroitinase ABC digestion (Figure 3.3B) in all samples examined. The disappearance of the electrophoresis bands of CS and DS standards and sulfated GAG polysaccharides from cartilage and skin after digestion indicates the occurrence of depolymerisation (Figure 3.3C: lanes 4, 6, 8 and 10, respectively). The hydrolysates have been ultra-filtrated through the membrane at 10 kDa MWCO to eliminate the undigested GAGs.

3.3.4. The effect of the enzymatic GAG depolymerisation on antioxidant activity

The sulfated GAG polysaccharides have been considered as a candidate for antioxidant supplement from a “natural” source (Shao, Chen, Pei and Sun, 2013). Biomolecular oxidation is a free radical mediated process. Antioxidants are effective in protecting living organisms against

oxidative damage by either direct scavenging of free radicals or the chelating of transition metals like Cu^{+} or Fe^{2+} that in turn are responsible for the initiation of Haber-Weiss and Fenton's reactions (Camp, Avenoso, Campo, Ferlazzo, and Calatroni, 2006). Variations in both the degree and position of the sulfate groups along the GAG polysaccharide chains not only contribute to the complexity of the GAG structure but are also found to be responsible for their bioactivity in terms of metal chelation and free radical scavenging (Prydz and Dalen, 2000; Volpi, 2006).

3.3.4.1. DPPH radical assay

The scavenging activities of sulfated GAG polysaccharides from cartilage and skin on DPPH radicals are shown in Figure 3.4A. The testing concentration was 1.0 mg/mL. As shown in Figure 3.4A, the DS and CS standard revealed scavenging activities on DPPH radical at 34 ± 9 and 38 ± 3%, respectively, which were significantly lower compared to ascorbic acid (79 ± 1%). The sulfated GAG polysaccharides extracted from chicken cartilage had less ($p < 0.05$) radical scavenging activity compared to the CS standard, but the skin sulfated GAGs were not different from both DS and CS standards (Figure 3.4A). This suggests that the negatively charged sulfated GAG polysaccharides had some DPPH radical scavenging activity probably due to their sulfate moieties attached to every disaccharide unit of their polysaccharide chains. As reported in the introduction, the sulfate groups would lead to a weaker dissociation energy of the hydrogen bonds among the polysaccharide chains promoting the donation of hydrogens from the hydroxyl groups (Wang et al., 2015).

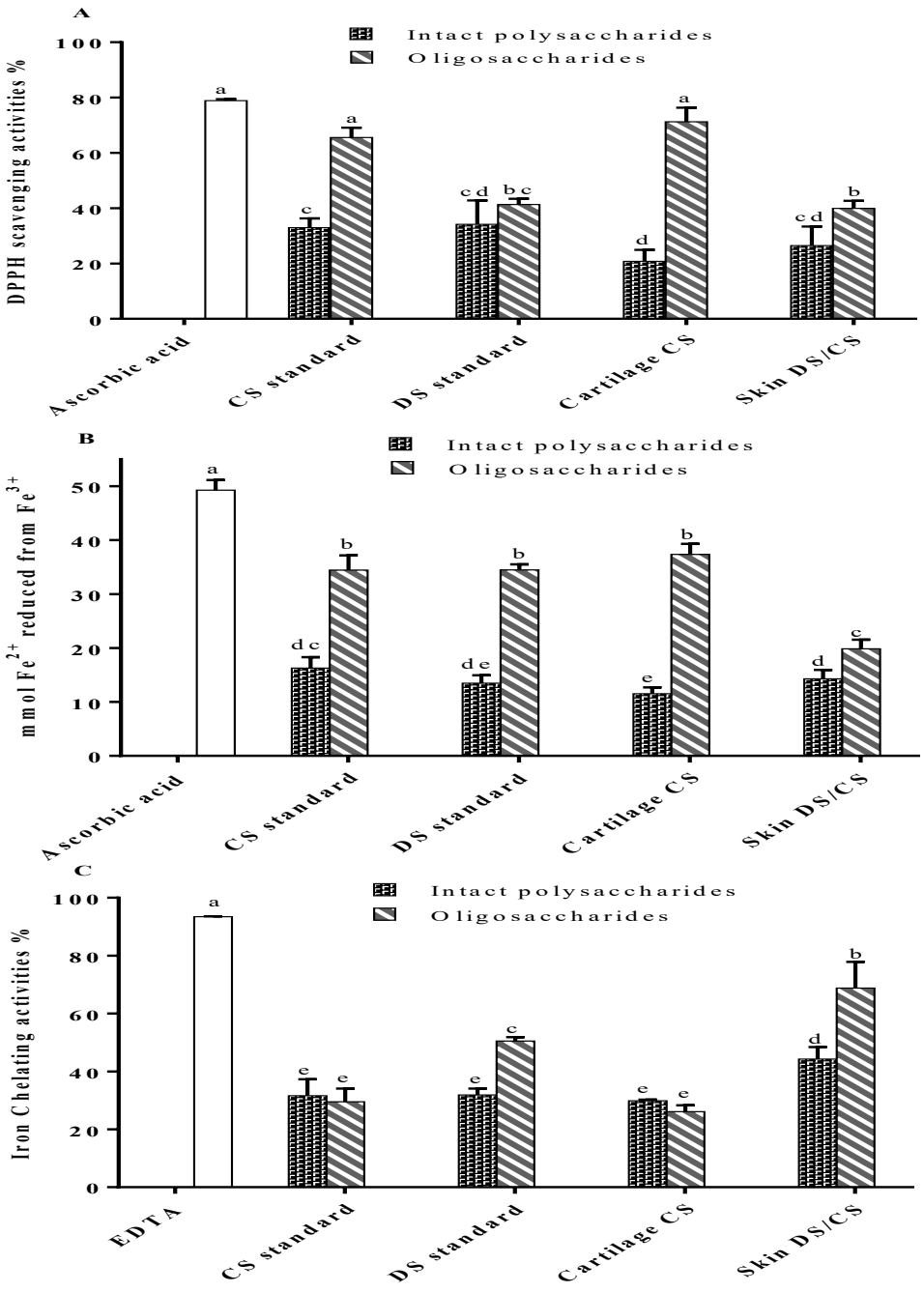

Figure 3.4. DPPH scavenging activities (A), iron reducing activities (B) and iron chelating activities (C) for the sulfated GAG polysaccharides and depolymerized sulfated GAG oligosaccharides from cartilage CS and skin DS/CS. CS from bovine trachea and DS from porcine intestinal mucosa were used as standard GAGs. Values are expressed as mean ± standard deviation (n=9); $p<0.05$. * sample concentration of 1.0 mg/ml; 50 mM of Fe3+ was used.

The DPPH scavenging activities were substantially improved by the depolymerisation treatment (Figure 3.4A). The CS oligosaccharides produced from the standard and the sulfated GAG oligosaccharides obtained from cartilage significantly increased the scavenging activities (81 ± 4 and 70 ± 4%, respectively) compared to their respective undigested and intact polysaccharide forms. Remarkably, these values were comparable with the ones obtained with ascorbic acid (79 ± 1%). This suggests that reducing the molecular weight of sulfated GAG polysaccharides is a critical step to improve their DPPH radical scavenging activity. Tang et al. (2014) also found that depolymerized polysaccharides with smaller molecular weights had a comparable scavenging effect to ascorbic acid but only at an increased sample concentration (5.0 mg/mL). The reason for this incremental effect of scavenging activity after depolymerisation may be due to the generation of more hydroxyl terminal groups during the cleaving process. The sulfated GAG oligosaccharides produced from skin also showed significantly improved scavenging activities (47 ± 4%) on the DPPH radical compared to their intact polysaccharide forms, but not as great as the sulfated oligosaccharides produced from cartilage.

3.3.4.2. Ferric (Fe^{3+}) reducing activity and iron chelating capacity

Ferric reducing activity is a single electron transfer method that is commonly used for antioxidant analysis in detecting the ability of a tested compound to transfer electrons, resulting in a color change when Fe^{3+} is reduced. It is important to know the reducing power of GAGs for the Caco-2 cell studies. As mentioned in the previous section, the ability to reduce Fe^{3+} to Fe^{2+} is the key for iron to be transported by the DMT-1 transporter on the apical surface of the epithelial monolayer. The Fe^{3+}– Fe^{2+} reduction in the presence of sulfated GAG polysaccharides and their oligosaccharide derivatives were investigated by their ferric reducing activities based on the ferrozine assay, a colorimetric method with a reagent that specifically binds to Fe^{2+} but not to Fe^{3+}

(Timmerman and Woods, 1999). With the total of 50 mmol Fe^{3+} initially added to the mixture, the ascorbic acid (positive control) was able to reduce 49 ± 2 mmol of Fe^{3+} to Fe^{2+} (Figure 3.4B). The amount of Fe^{2+} reduced from Fe^{3+} was significantly less ($p < 0.05$) in the extracted sulfated GAG polysaccharides as well as in the CS and DS standards compared to ascorbic acid. By contrast, after the depolymerisation, all the sulfated oligosaccharide samples showed substantial improvement in their ferric reducing activities ($p < 0.05$). Among these treatments, the sulfated oligosaccharides produced from both CS and DS standards as well the ones produced from cartilage showed the greatest increase of Fe^{2+}. The sulfated GAG oligosaccharides from skin also had greater ($p < 0.05$) reducing activities compared to their polysaccharide counterparts. These data, once again, confirm the importance of the depolymerisation step for increasing the antioxidant capacity of sulfated GAGs. As highlighted in the previous section about the radical scavenging activity, the generation of more hydroxyl terminal groups could also contribute to the capacity of the sulfated GAG oligosaccharides to donate electrons.

The ability to bind transition metals is another important indicator for evaluating the antioxidant properties of the natural compounds. The capacity to chelate transition metals decreases their availability to act as catalysts for initiating radical mediated oxidative chain reactions in biological or food systems. For instance, the iron chelating agents inhibit the Fenton reaction and hydroperoxide decomposition, thus reducing the generation of the reactive oxygen species. The capacity of sulfated GAGs to bind positively charged transition metal ions, such as Cu^{2+} and Fe^{2+}, has been widely accepted and reported by many researchers (Volpi and Tarugi, 1998; Volpi, 2006). Also, the binding/chelation property seems to be an important factor to increase the transportation of iron within the Caco-2 cell monolayer through endocytosis of GAG-iron complexes (Laparra et al., 2009). The ferrous chelating activity is reported in Figure 3.4C and expressed as the percentage

of inhibition. Greater inhibition indicates a greater iron binding capacity of the test sample. None of the sulfated GAG polysaccharides showed iron chelating activities above 50%, with the cartilage GAGs showing the least activity of 26.1 ± 2.2%. After enzymatic depolymerisation, both DS and skin sulfated oligosaccharides showed a significant increase in their iron chelating capacities at 51 ± 1 and 69 ± 9%, respectively. The increasing trends were not observed for the depolymerized CS standard and cartilage sulfated GAGs (Figure 3.4C). In summary, the depolymerisation is an important step to increase the reducing capacity of the sulfated GAGs from each chicken tissue tested in this experiment, while the effect of depolymerisation on the chelation activity was evident only in the sulfated GAGs extracted from skin.

3.3.5. Effect of the enzymatic depolymerisation on ferritin formation in human intestinal Caco-2 cells

The use of *in vitro* digestion/Caco-2 cell culture model is an established method to evaluate non-heme Fe uptake through ferritin formation (Glahn et al., 1998; Laparra et al., 2008; Laparra et al., 2009; Xiao et al., 2014). The gastrointestinal digestion is a required step to initiate the promoting effects on Fe uptake by the Caco-2 cells and also to closely mimic the physiological environment of the human digestion system. In the lumen, the solubility of iron and the formation of soluble ferrous complexes (Scheers, Andlid, Alminger and Sandberg, 2010) are the main determinant of Fe available for uptake into the enterocytes. Caco-2 cells spontaneously differentiate to develop characteristics of small intestinal enterocytes, which contain all the relevant uptake and transport proteins for iron. Glahn et al. (1998) reported that Caco-2 monolayers are very sensitive to the available iron and have exhibited a maximal absorption capacity when exposed to Fe concentrations between 20 and 50 μM. In the present study, the concentrations of ascorbic acid (positive control) and Fe^{3+} (negative control) were based on previous studies (Laparra, et al., 2008; Lappara et al., 2009). Results about ferritin formation in the monolayers are

shown in Figure 3.5. The treatment with the sulfated GAG polysaccharides extracted from chicken cartilage and skin induced greater ($p < 0.05$) ferritin formation than the addition of $FeCl_3$ alone (100 ± 4 and 88 ± 4 ng/mg protein, respectively vs 37 ± 1 ng/mg protein, $p < 0.05$). This indicates an enhancing effect of sulfated GAG polysaccharides extracted from chicken tissues on Fe uptake by Caco-2 cells. Remarkably, the GAG-oligosaccharides produced from both CS standard and the chicken cartilage showed an increased formation ($p < 0.05$) of ferritin by almost two-fold compared to their intact forms. Sulfated GAG oligosaccharides from skin also improved ($p < 0.05$) the ferritin formation compared to their intact polysaccharides counterpart, but at a significantly lower level compared to the ones obtained from commercial CS and cartilage. Results reported in the previous section indicated that the sulfated GAG oligosaccharides from chicken cartilage increased their DPPH radical scavenging activity and ferric reducing activity, but not the iron chelating activity (Figure 3.4). These results seem to indicate that the enhancing effect on Fe uptake is mainly affected by the reducing capacity of the GAG oligosaccharides rather than the metal chelation ability. In addition, the main problem affecting the efficacy of CS is likely its poor intestinal absorption due to its large molecular weight and charge density (Baici et al., 1992). The depolymerisation step also provides a means of producing lower molecular weights CS (< 10 kDa) that may improve their absorption by the Caco-2 cells. Xiao et al. (2014) had demonstrated that low molecular weight (17.5 kDa and 4.5 kDa) CS can be more readily transported across Caco-2 cell monolayers than its intact form. The sulfated GAG-mediated Fe uptake promoting effect has been previously reported by Laparra et al. (2008) with purified sulfated GAGs from cooked fish. Hurrell, Reddy, Juillerat and Cook (2006) also mentioned the possibility of the non-protein enhancer for Fe absorption from chicken protein isolates and suggested that GAGs are a potential candidate.

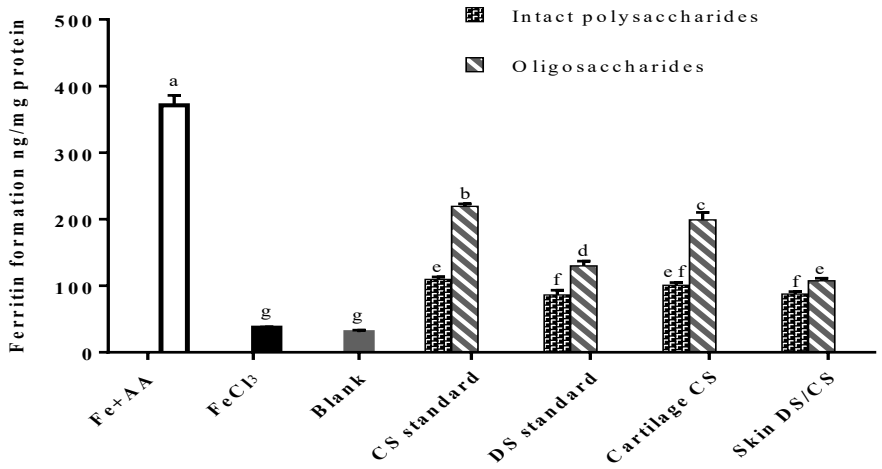

Figure 3. 4. Ferritin levels in Caco-2 cells exposed to the sulfated GAG polysaccharides and depolymerized sulfated GAG oligosaccharides from chicken skin and cartilage. Samples (final concentration=0.8 mg/mL) were mixed with $FeCl_3$ (final concentration=41.7 µmol/L) and subjected to *in vitro* digestion. Values are expressed as mean ± standard deviation (n=9 with three independent cell trails). AA: ascorbic acid.

3.4. Conclusion

The purification and identification of the sulfated GAG polysaccharides from chicken skin and cartilage tissues demonstrated that the main GAG in the chicken cartilage is CS, and that the majority of skin sulfated GAGs were present as CS/DS copolymers. The antioxidant capacity of the sulfated GAG polysaccharides was greatly improved by enzymatic depolymerisation creating sulfated oligosaccharides. Caco-2 cell ferritin formation was also enhanced with the addition of sulfated GAG oligosaccharides from chicken cartilage. This could be due to the combined effects of reducing the molecular weight of CS polymers by enzymatic hydrolysis and increasing the ferric reducing activity. The results not only confirm that sulfated GAGs are responsible for the enhancing effect of the inorganic iron absorption, but also suggest a possible means to increase this effect. Indeed, since the use of costly enzymes may limit the possibility of implementing such a technology in a commercial context, new and practical depolymerisation approaches should continue to be evaluated.

Chapter 4. Supplementation of chondroitin sulfate-oligosaccharide in skim bovine milk improves Fe uptake in a human intestinal Caco-2 cell line[2]

4.1. Introduction

Iron deficiency is one of the most prevalent nutritional disorders in the world, affecting mostly young children and women of child-bearing age, with approximately 1/4 of the world's population being iron-deficient (Mclean, Gogswell, Egli, and Benoist, 2008). The World Health Organization recommends exclusive breastfeeding without supplementation with bovine milk for 6 months (Kramer and Kakuma, 2012) to provide complete nourishment for the newborn and avoid iron deficiency during infancy. Although bovine milk has a similar composition of protein, fat, lactose, vitamins and minerals, there is strong evidence suggesting human milk not only provides adequate nutrients, but also contains factors that may facilitate the absorption of minerals, especially iron through human intestinal wall (Argyri, Miller, Glahn and Kapsokefalou, 2007; Etcheverry, Wallingford, Miller and Glahn, 2004; Etcheverry, Miller and Glahn, 2004). Despite milk being the main food source during early life, this food has very little iron from a nutritional point of view, therefore extrinsic iron supplementation is required for the growth of infants after 6 months (Ziegler, Nelson and Jeter, 2009). The fortification of bovine milk or infant milk formulas with iron is a widely accepted strategy, but the absorption of this non-heme iron is poor. Iron supplementation of milk may also negatively affect its organoleptic properties and stability. Moreover, from a nutritional point of view, since iron bioavailability is more important than the total iron content (Ziegler et al., 2009), strategies that promote iron bioavailability are more effective than simply increasing the total amount of iron in a food matrix. The enhancing effect of human milk on non-heme iron absorption has also been studied. These studies show that its Fe

[2] *A version of this chapter has been published in the Journal of Functional Food. (Wang H., and Betti M. 2018. Journal of Functional Food, 46:556-66.)*

absorption enhancing effect is attributed to the combination of the intrinsic Fe levels, lactoferrin, ascorbic acid, lactose and cysteine, all of which occur in greater concentrations in human compared to bovine milk (Argyri et al., 2007; Etcheverry et al., 2004; García-Nebot et al., 2010; Kibangou et al., 2005).

However, little research has been done to understand the effect of milk glycans (such as oligosaccharides, glycoconjugates and glycosaminoglycans), and other minor milk components on iron absorption. Only in recent years has it become apparent that this carbohydrate fraction in milk, whether free or bound to proteins, plays essential roles in many inter- and intracellular processes (Urashima, Taufik, Fukuda and Asakuma., 2013; Coppa et al., 2011; Bode, 2012; Coppa et al., 2013). In particular, bovine milk oligosaccharides were recently identified as being structurally similar to human milk oligosaccharides and these may confer similar benefits to human health (Zivkovic and Barile. 2011; Bode, 2012; Martinez-Ferez et al., 2006). As for glycosaminoglycans (GAGs), chondroitin sulfate (CS), dermatan sulfate (DS), heparin and heparan sulfate were found in both types of milk. However, GAGs concentration in human milk were shown to be about 7 times greater than that in bovine milk, especially regarding CS, which represents as much as 55% of the total GAGs in human milk, but only 21% in bovine milk (Coppa et al., 2011). Interestingly, Laparra, Barbera, Alegria, Glahn, and Miller (2009) demonstrated that the presence of CS/DS-related structures have a positive effect to increase the Fe uptake by Caco-2 cells. Moreover, in the study described in chapter 3, enzymatically depolymerized GAG-oligosaccharides further improved the Fe uptake by the *in vitro* digest/Caco-2 cell culture model, where the CS-oligosaccharides (CS-oligos) showed the greatest enhancement on ferritin formation (chapter 3). The difference in the content of CS-related structures between human and bovine milks is likely responsible for most of the difference in their Fe-uptake enhancing effects. Thus, bovine CS-oligos

are a reasonable candidate to supplement in Fe-fortified bovine milk or infant formulas to improve the Fe bioavailability in humans, since CS is the major GAG, which is lacking in bovine milk compared to human milk. This assumption has very practical applications to improve global human nutrition regarding iron status. Bovine milk is a common staple used worldwide in nutritionally balanced diets and it is usually consumed with Fe-fortified food (i.e. cereals). Supplementing bovine milk with CS-oligosaccharides to promote Fe uptake may provide a better means to overcome its inadequate bioavailability on non-heme iron and would complement an extrinsic iron supplementation strategy.

The main objective of this study was to determine whether the CS-oligos supplementation in skim bovine milk (SBM) can indeed improve iron bioavailability. It is important to understand how it may interact with the normal existing components of Fe inhibitors and enhancers in bovine milk. Here, iron bioavailability was determined by measuring ferritin formation in the *in vitro* digestion/Caco-2 cell model system. Extrinsic iron was added to represent an exogenous iron source from the diet. The SBM was fractionated into casein, whey, lactose and milk oligosaccharides components. GAGs in the milk oligosaccharides fraction were extracted and characterized using cellulose acetate electrophoresis, enzyme digestion liquid chromatography fluorescence detection and electrospray ionization mass spectrometry (HPLC-FLD-ESI-MS). Furthermore, in order to understand which component of the SBM had an enhancing or inhibiting effect on Fe uptake, fractions such as “SBM minus lactose”, “SBM minus casein” etc. were also produced and supplemented with the Fe and CS-oligos.

4.2. Materials and Methods

4.2.1. Materials and reagents

SBM (2 L) was purchased from a local supermarket (Dairyland, Saputo Inc., BC, Canada). Chondroitin sulfate sodium salt (70% chondroitin sulfate A; 30% chondroitin sulfate C) from bovine trachea, 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*, hyaluronic acid from rooster comb, chondroitin sulfate B (DS) from porcine intestinal mucosa, chondroitin disaccharide standards (Δdi-0S, Δdi-4S and Δdi-6S), pancreatic enzymes from porcine pancreas, pepsin from porcine gastric mucosa and bile extract were obtained from Sigma–Aldrich (Mississauga, ON, Canada). Chelex-100 was from Bio-Rad Laboratories (Hercules, CA, USA). Diethylaminoethyl (DEAE) cellulose anion exchanger (DEAE Sephacel) was obtained from GE Healthcare, Bio-Science (Mississauga, ON, Canada). 2-Aminoacridone (AMAC, >98%), glacial acetic acid, dimethyl-sulfoxide (DMSO, 99.9%), and sodium cyanoborohydride (95%) and all other reagents, of the highest purity available, were from Sigma-Aldrich. All solutions were prepared with Milli-Q purified water (17 MΩ·cm, Millipore, Bedford, MA, USA).

4.2.2. Skim bovine milk fraction preparation

The SBM was fractionated as described by Martinez-Ferez et al. (2006) with some modifications indicated in Figure 4.1A. SBM was subjected first to ultrafiltration (Minimate^{TM} TFF system, Pall Corporation, MI, USA) using a capsule with a 10 kDa molecular weight cut-off (MWCO) for 16 h at 4°C, and the retentate containing skim milk proteins fraction was collected and saved for later separation. The filtrate, a lactose-containing fraction, was subjected to the second ultrafiltration with a 1 kDa MWCO capsule. The filtrate, containing lactose and salts were freeze-dried and saved. The retentate, a low molecular weight milk fraction containing some

soluble polypeptides, oligosaccharides, and glycoconjugates was considered as the bovine milk oligosaccharides fraction. The skim milk protein fraction was warmed at 40˚C, to which 60% acetic acid was added drop-wise until the casein started to coagulate and precipitate formed. The casein pellet was collected after centrifugation at 11,300 × *g* at 4°C for 20 min using an Avanti J-E centrifuge (Beckman Coulter Inc., Palo Alto, CA, USA.) and freeze dried for later analyses. The supernatant portion ("whey") was also freeze-dried and saved for later analyses.

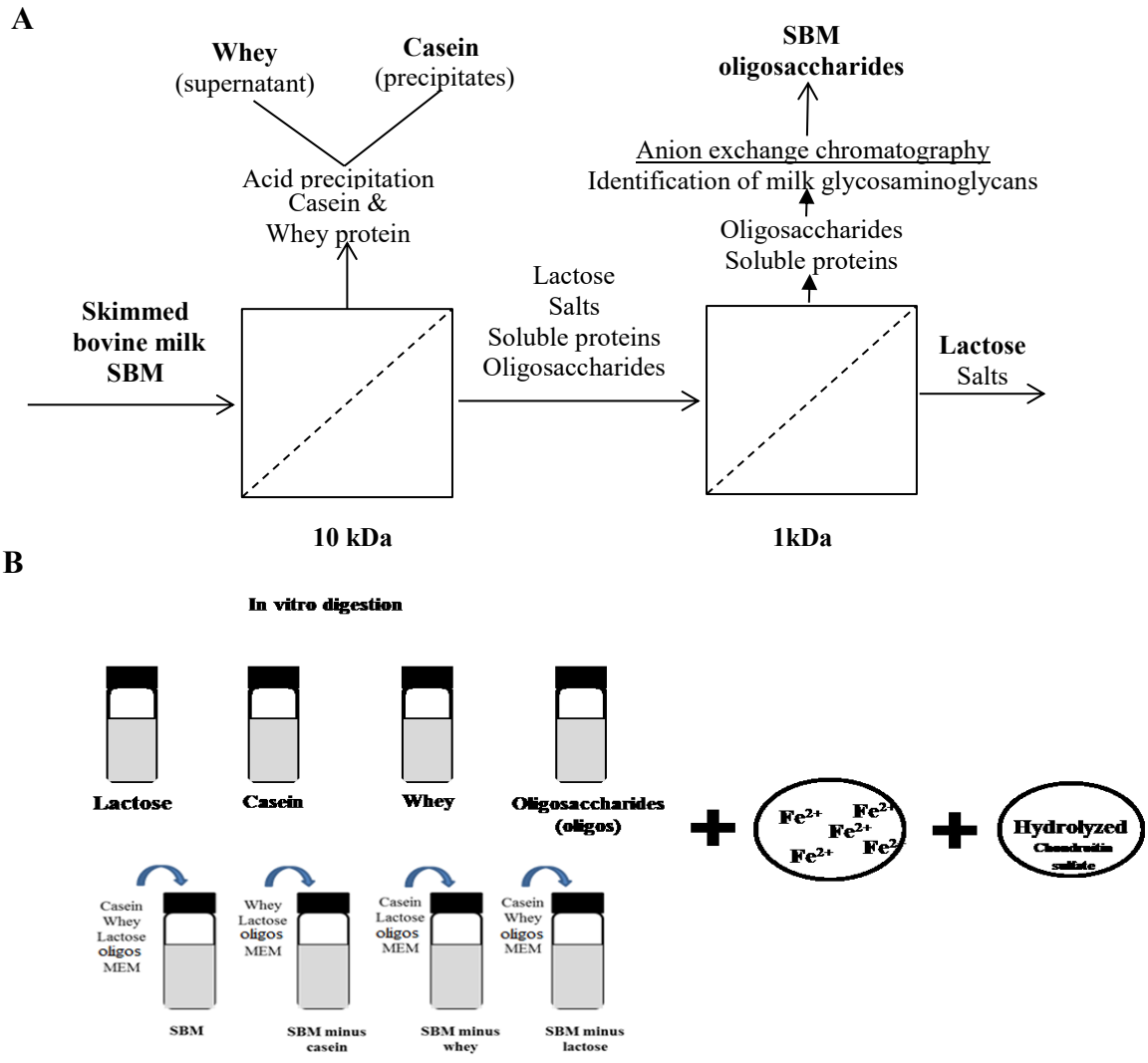

Figure 4. 1. A) Scheme of the two-stage ultrafiltration process for the separation of milk component with a filtration molecular weight cut-off at 10 and 1 kDa; B) Fractionation procedure for skim bovine milk (SBM). MEM = minimum essential medium. Oligos = oligosaccharides.

To obtain SBM minus lactose fraction, the lactose was removed after the second ultrafiltration and put into a pre-weighed test tube and replaced with an equal weight of minimum essential medium (MEM; GIBVO). To prepare SBM minus casein fraction, the lactose containing fraction and the whey fraction were transferred to a clean test tube. The casein pellet was weighed and an equal amount of MEM was then added. To prepare SBM minus whey fraction, the casein and lactose containing fractions were combined, and MEM was added to replace the whey. All modified SBM fractions were freeze-dried and saved for later analyses (Figure 4. 1B).

4.2.3. Purification of bovine milk GAGs

The low molecular weight milk oligosaccharides fraction was freeze-dried and purified by deproteinization and anion exchange chromatography according to the previous chapter. After proteolysis with pancreatic enzyme, three volumes of 100% ethanol were added to completely precipitate GAGs. The precipitated GAGs were then dissolved in deionized water and applied to an anion exchange column (1.5 cm × 5 cm) of DEAE cellulose, which was equilibrated with water adjusted to pH 3.0 with HCl. The column was eluted with a linear gradient formed from 25 mL of water, 25 mL of 0.4 M NaCl and 25 mL of 1.0 M NaCl all adjusted to pH 3.0. Elution was further continued with 70 mL of 2 M NaCl. The fractions that were positive to the uronic acid assay carbazole reaction (Kosakai and Yoshizawa, 1979) were collected for further characterization and the GAG elution chromatogram was generated. The total GAGs from the skim bovine milk were also extracted by the same method described above. The content of GAGs in the milk oligosaccharides fraction and skim bovine milk were calculated using a factor of 3 to convert from uronic acid content according to Nakano et al. (1996).

4.2.4. Determinations of iron in SBM fractions

The intrinsic iron concentration in each milk fraction was measured by the colorimetric ferrozine assay according to Viollier et al. (2000) with minor modifications. Briefly, the reaction mixture containing sample (10 mg/mL), ferrozine (10 mM), reducing agent (1.0 M ascorbic acid) and buffer (2.5 M ammonium acetate, pH 9.5) was adjusted to a total volume of 0.2 mL and distributed into a microplate. The plate was shaken vigorously and incubated at room temperature for 30 min and the absorbance was measured at 562 nm with a microplate reader (SpectraMax M3, Molecular Devices, Sunnyvale, CA, USA). All sample fractions were diluted 10 times with MEM before the extrinsic iron source was added and the iron content was measured again to make sure the amount of iron in each fraction was relatively the same.

4.2.5. Cellulose acetate electrophoresis of skim bovine milk GAGs from the oligosaccharide fraction

The bovine milk GAGs and dermatan sulfate were digested with chondroitinase-ABC and chondroitinase-ACII with 5.0 μg uronic acid/0.03 unit of enzyme and 1.0 mg uronic acid/0.005 unit of enzyme, respectively, in 0.01 M sodium acetate buffer containing 0.02% sodium azide at 37°C for 1 h. The pH of the buffer was 8.0 for chondroitinase-ABC and 6.0 for chondroitinase-AC (Nakano et al., 1996). Electrophoresis on cellulose acetate strips was carried out for all samples (1.0 mg/mL) before and after the chondroitinase digestions in 0.1 M pyridine/0.47 M formic acid at pH 3.0 according to Hata and Nagai (1971). After electrophoresis, each strip was stained in 0.1% (w/v) Alcian blue 8GX in 0.1% acetic acid and washed extensively with deionized water.

4.2.6. Derivatization of bovine milk GAGs from oligosaccharide fraction with AMAC

The chondroitinase-ABC and chondroitinase-ACII digested bovine milk GAGs and dermatan sulfate samples were obtained from Section 4.2.5. The derivatization of the Δ-disaccharide

standard and the samples were performed as previously described by Coppa et al. (2011) with minor modifications. AMAC, a well-known fluorescent hydrophobic molecule successfully used for the derivatization and separation of unsaturated (oligo) disaccharides, was also used in this study. The sample was reconstituted with 5 μL of a 0.1 M AMAC solution in glacial acetic acid: DMSO (3:17, v/v) and 5 μL of a freshly prepared solution of 1.0 M sodium cyanoborohydride in water. Then, the mixtures were centrifuged in a microfuge at 11,000 x *g* for 3 min. Derivatization was performed by incubating at 45˚C for 4 h. Finally, 190 μL of 50% v/v DMSO were added to the samples and aliquots were taken for HPLC-FLD-ESI-MS analyses.

4.2.7. Liquid chromatography and mass spectrometry analyses of AMAC-derivatives

HPLC-FLD-ESI-MS was performed using an Agilent 1200 SL HPLC System with a Glycan PAC AXH-1 hydrophilic interaction (HILIC) column, 2.1x150 mm, 1.9 μm particle size (Thermo Scientific, Sunnyvale, CA, USA), with precolumn at 40°C. The buffer gradient system was composed of 100 mM ammonium formate in water, pH 4.45, as eluent A and acetonitrile as eluent B. An aliquot of the sample was loaded onto the column at a flow rate of 0.4 mL/min. An initial buffer composition was 90% eluent A and 10% eluent B and the column was washed for 0.5 min under these conditions. The column was eluted with a linear gradient from 90% to 75% eluent B over a period of 0.5 min, and 75% to 45% eluent B for 9 min, kept at 45% eluent B for 1.5 min and returned to 90% eluent B for 0.5 min. Flow was split at 50:50 between fluorescence and MS detectors. Fluorescence signals used were 425 nm for excitation and 520 nm for emission. Mass spectra were acquired in negative mode of ionization using an Agilent 6220 Accurate-Mass HPLC/MS system (Santa Clara, CA, USA) equipped with a dual sprayer electrospray ionization source with the second sprayer providing a reference mass solution. Mass spectrometric conditions were drying gas 10 L/min at 325°C, nebulizer 20 psi, mass range 100-3200 Da, acquisition rate of

~1.03 spectra/s, fragmentor 140V, skimmer 65V, and capillary 3800V. Data analysis was performed using the Agilent MassHunter Qualitative Analysis software package version B.07.01. The disaccharides were determined by retention time and *m/z* values were calculated according to the single charged mode that was used.

4.2.8. Preparation of chondroitin sulfate oligosaccharides (CS-oligos)

Chondroitin sulfate A sodium salt from Sigma–Aldrich (Mississauga, ON, Canada) was digested with testicular hyaluronidase according to the method reported by chapter 3.

4.2.9. Cell culture

The Caco-2 cells were purchased from the American Type Culture Collection (Rockville, MD., USA) and used in all experiments described in this study between passages 22–24. Stock cultures were maintained in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Grand Island, NY, USA), pH 7.4, supplemented with 20% (v/v) fetal bovine serum (GIBCO), 5% HEPES and 1% antibiotic antimycotic solution (GIBCO). The cell culture procedure is referred to section 3.2.9. in chapter 3.

4.2.10. In vitro digestion

In vitro digestion was developed by Glahn et al. (1998) to mimic the human gastrointestinal digestion process. All individual SBM fractions (casein, whey, lactose and oligosaccharides) as well as SBM, SBM minus casein, SBM minus whey, SBM minus lactose with and without CS-oligos samples were subjected to the process of gastrointestinal digestion. Samples were diluted in 1:10 ratio with MEM. This dilution was necessary to even out the intrinsic Fe level. Extrinsic iron (as ferrous sulfate) was added to some samples at the concentration of 20 µmol/L. The final concentration for the milk samples was 1 mg/mL and the final concentration of CS-oligos added

was 0.5 mg/mL (based on the MTT cell proliferation assay). Each sample was digested by simulated gastrointestinal digestion using two demineralized enzymatic solutions (pepsin solution at pH 2 for 1 h at 37˚C, and pancreatin-bile solution at pH 6.5 for 2 h at 37˚C) as previously described (chapter 3). The baseline cell ferritin was measured as the blank. All samples were prepared on the same day and there were three independent replications.

4.2.11. Ferritin analysis

Ferritin analysis assays is referred to section 3.2.11 in chapter 3.

4.2.12. Statistical analyses

Each treatment was performed in triplicate. Data were subjected to the tow-way analysis of variance (ANOVA) and the significance of the differences between treatments was determined using Tukey test with $p < 0.05$.

4.3. Results and discussion

4.3.1. Skim bovine milk composition

Milk is traditionally recognized as an excellent source of nutrients. The focus of most reviews on the nutritional properties of milk has been on its contribution of fat, calcium and proteins. These compounds may have negative or positive health effects depending on the perspective and circumstances (Huang, Hostmark and Harstad. 2007). The recent research and meta-analyses had evidence suggests that milk has a neutral effect on cardiovascular outcomes but fermented dairy products such as yogurt, kefir and cheese may have a positive or neutral effect (Lordan et al., 2018). However, the dietary trends indicate that generally there is a reduction of full-fat diary product consumption and increased low-fat dairy consumption (Lordan et al., 2018). In addition, Etcheverry, Miller and Glahn (2004) reported that bovine milk fat may contribute to the inhibitory

effect on the Fe uptake by Caco-2 cells. In the current study, SBM was used as the starting material and subsequent fractionations were performed according to Figure 4.1A. The concentration of each component in the SBM is listed in Table 4.1. According to Guetouache, Bettache, and Sanur (2014), SBM contains about, 48 g/L lactose, 28 g/L casein and 6.4 g/L whey, which are similar to the results from this study. The SBM oligosaccharides fraction was separated from the lactose fraction (Figure 4.1A) with a MWCO of between 1 to 10 kDa at a concentration of 125 ± 7 mg/L. This agrees with the results reported by Urashima et al. (2013). The SBM oligosaccharides fraction were deproteinated and eluted through an anion exchange column with an increasing NaCl gradient. The collected fractions were positive for uronic acid, indicating the presence of GAGs in this relatively low molecular weight fraction of milk. In mammalian connective tissues, the molecular weight of CS usually ranges from 20-50 kDa. The calculated GAGs content was 41 ± 3 mg/L and represented about 33% (w/w) of the SBM oligosaccharides collected. This suggests that the GAGs collected from this fraction were likely from the low molecular weight bovine glycoproteins. On the other hand, the total GAGs concentration in SBM was 67 ± 1 mg/L. There are limited studies available for GAGs content from the bovine source of milk. Coppa et al. (2013) reported that the total amount of GAGs in mature bovine milk is around 30–60 mg/L. More importantly, about 62% of GAGs were identified in the low molecular weight milk oligosaccharide fraction. Bovine milk glycan has been an overlooked component in studies that relate to the effect of bovine milk on iron availability. This is likely due to its negligible proportion compared to the other milk components. In addition, most of the studies have not separated carbohydrate containing components (lactose and oligosaccharides) from the soluble whey fraction; this raises the question if these soluble milk components also contribute to the promoting effect on iron absorption that researchers reported for the low molecular weight whey fraction (Etcheverry, Miller, and Glahn,

2004; Agryri et al. 2007). Therefore, in this study, both lactose and oligosaccharides have been purposely separated from the milk protein fractions. The detection of GAGs in the low molecular weight milk oligosaccharide fraction is the first to be reported in this study; further characterization is discussed later.

Table 4. 1. Concentration of fractionated skim bovine milk components.

Fractionated skim bovine milk components	Concentration
Bovine milk proteins	
Casein (g/L)	26 ± 3
Whey (g/L)	6 ± 1
Bovine milk carbohydrates	
Lactose (g/L)	52 ± 4
Oligosaccharides (mg/L)	125 ± 7
Bovine milk glycosaminoglycans (GAGs)	
Total GAGs (mg/L)	67 ± 1
GAGs from oligosaccharides fraction (mg/L)	41 ± 3
Iron (μmol/L)	3.9 ± 0.5

4.3.2. Iron content in the SBM fractions and in vitro digestion of milk proteins

The intrinsic iron concentration in the SBM was 3.9 ± 0.5 μmol/L, which is on the low end of the 4.5 to 13.4 μmol/L Fe range reported for bovine milk (Etcheverry, Miller and Glahn., 2004). The intrinsic Fe concentration in each SBM fraction is presented in Table 4. 2. The greatest concentration of Fe was in the lactose fraction and the least was in the casein fraction (10.6 ± 1.4 μmol/L and 0.71 ± 0.2 μmol/L, respectively). The greater iron content in the lactose fraction is probably due to the fractionation process, since most of the minerals including iron have molecular weights less than 1 kDa and thus are filtered through the membrane with the lactose. After dilution of the sample, the intrinsic iron content in each fraction was reduced and the final total iron concentration with the addition of 20 μmol/L extrinsic iron source was in a range of 20 to 21 μmol/L (Table 4.2). In this way, the intrinsic Fe content in SBM was minimized and the final iron concentration in each sample was relatively the same.

Table 4. 2. The iron concentration in each skim bovine milk fraction

Skim Bovine milk	Intrinsic Fe[1]	Final total Fe[2]
Components	μmol/L	
Casein	0.7 ± 0.2	20.04 ± 0.03
Whey	2.5 ± 0.5	20.12 ± 0.02
Lactose	10.6 ± 1.4	20.53 ± 0.07
Oligosaccharides	1.4 ± 0.2	20.07 ± 0.01
SBM[3]	3.9 ± 0.4	20.20 ± 0.03

[1]Intrinsic iron concentration in the fractions before the 1:10 dilution with minimum essential medium (MEM);
[2]Total iron in each fraction after dilution of the fractions with MEM, then adding 20 μmol/L Fe;
[3]SBM=skim bovine milk.

Milk contains two major classes of protein: casein and whey, both of which influence iron availability. Milk peptides formed during enzymatic digestion have recently attracted more research attention because they exhibit a range of biological effects including opiate, antithrombotic, antioxidant, immunomodulatory and metal-binding properties (Bhat, Kumar and Bhat, 2015). In this study, the *in vitro* digestion was used to mimic the gastrointestinal digestion of the milk components. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to visualize the protein profile of casein and whey before and after the *in vitro* digestion (Appendix C). Electrophoresis indicated that after the *in vitro* digestion, casein still showed bands at α- and β-casein, suggesting that the casein was not completely digested. On the other hand, the main whey proteins, β-lactoglobulin and α-lactalbumin, showed some resistance to gastrointestinal digestion. No bands under 10 kDa were found in the SDS-PAGE after the digestion of both casein and whey. This suggests that the *in vitro* digestion did not completely digest the milk proteins into small molecular weight peptides (MW < 10 kDa). In this study, both casein and whey fractions

likely contained a mixture of large molecular weight polypeptides after the *in vitro* gastrointestinal digestion.

4.3.3. Identification of GAGs collected from the milk oligosaccharides fraction

In general, GAGs are linear heteropolysaccharides composed of a variable number of repeating disaccharide units, which usually are classified into two categories: non-sulfated GAGs (i.e. HA) and sulfated GAGs (i.e. CS and DS). DEAE cellulose anion exchange chromatography (Figure 4.2) of the milk oligosaccharides fraction digest showed two major uronic acid containing peaks eluting at 0.4 M and 2.0 M NaCl. The two fractions were combined in this study for milk GAGs characterization.

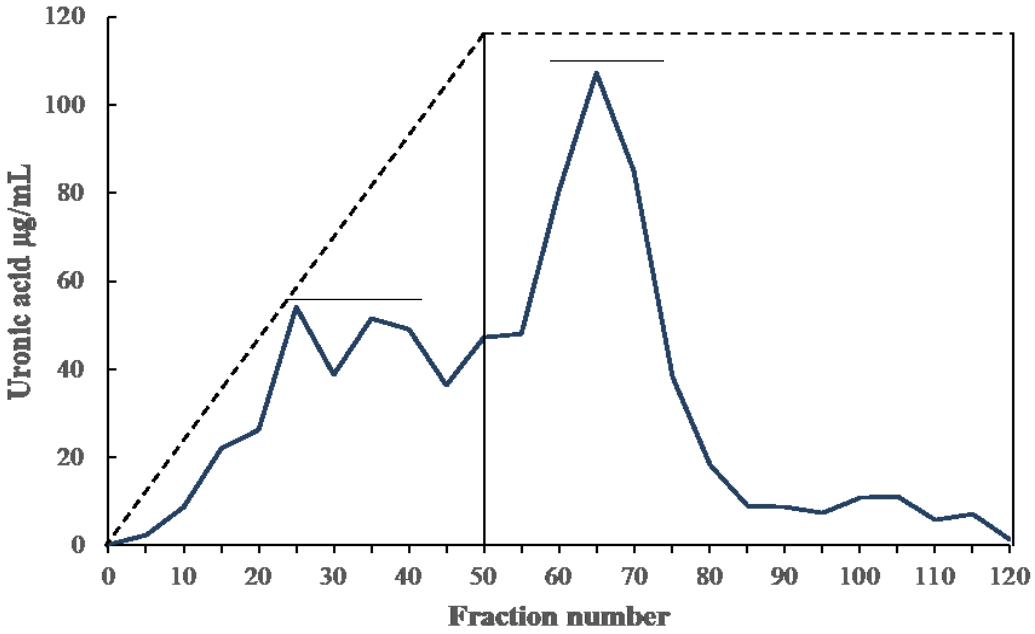

Figure 4. 2. Chromatography of bovine milk oligosaccharides fraction on a column of DEAE-cellulose. Fractions (1 mL) collected at a flow rate of 0.5 mL/min were monitored for uronic acid (µg/mL) by the carbazole reaction. Fractions 0-50 contained eluates with 0.4-1.0 M NaCl gradient and those 50-120 contained eluates with 2.0 M NaCl. Bars denote fractions pooled for further analysis. ------ NaCl gradient.

Cellulose acetate electrophoresis of the extracted milk GAGs gave a broad band (Figure 4.3: lane 4) with its fast migrating front possessing a mobility similar to that of CS (Figure 4.3: lane 1), but its densely stained portion had a mobility less than CS, but similar to that of DS (Figure 4.3: lane 2). The lower part of the band had slower mobility between the positions of DS and HA (Figure 4.3: lanes 2 and 3). The band of milk GAGs was completely susceptible to chondroitinase- ABC (band disappeared) but not chodroitianse-AC (Figure 4.3: lane 5 and lane 6). The chondroitinase-AC digest of milk GAGs had a narrow band with its mobility slightly slower than that of DS control standard band (Figure 4.3: lane 8). This suggests the presence of repeating disaccharide units of CS and DS. Nakano and Ozimek (1999) had reported a similar GAGs profile in the 2.0 M NaCl fraction with a combination of CS, DS and heparan sulfate. No heparan sulfate was identified in the milk GAGs extracted in this study probably since heparan sulfate is present as a major GAG in the bovine milk fat globule membrane (Newburg, Linhardt, Ampofo and Yolken. 1995), and thus may not be found in the milk oligosaccharide fraction obtained from the skim milk.

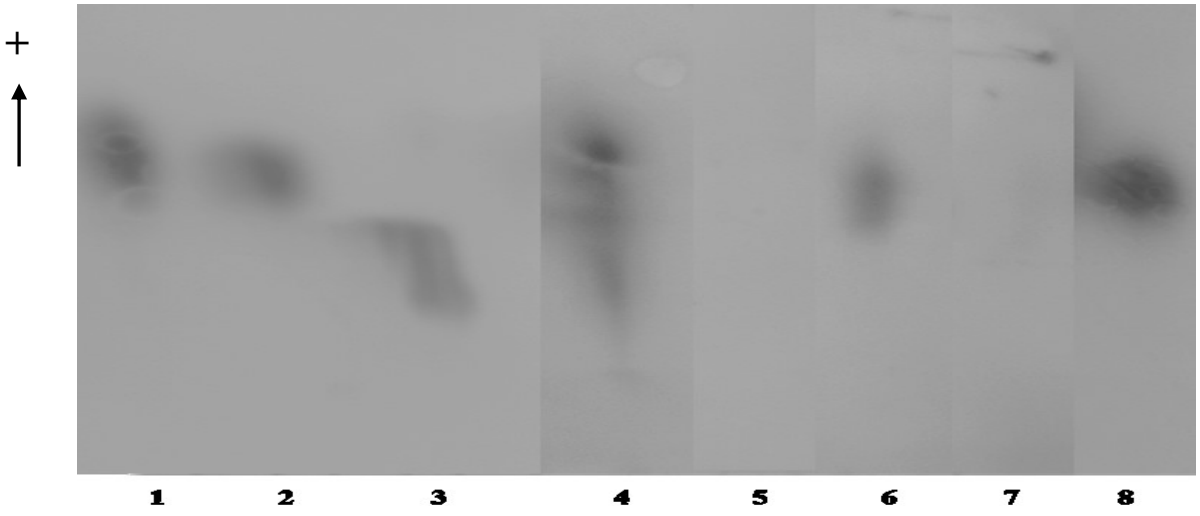

Figure 4. 3. Cellulose acetate electrophoresis of the bovine milk GAGs from the bovine milk oligosaccharides fraction. Lane 1: CS standard; Lane 2: DS standard; Lane 3: HA standard; Lane 4: GAGs from the bovine milk oligosaccharides fraction; Lane 5: bovine milk GAGs digested by chondroitinase-ABC; Lane 6: bovine milk GAGs digested by chondroitinase-AC; Lane 7: DS digested by chondroitinase-ABC. Lane 8: DS digested by chondroitinase-AC. The arrow indicates the direction of the mobility.

According to the CS and DS composition and the position of the sulfate group, three major Δ-disaccharides (Δdi-0S, Δdi-4S and Δdi-6S) are usually used as indicators to determine the disaccharide composition in CS and DS. In this study, HPLC-FLD-ESI-MS was performed to separate and structurally characterize the disaccharides from the chondroitinase-ABC and -AC digests of milk GAGs samples, and DS was used as a control. Chondroitinase-ABC and -AC are specific chondroitinase enzymes. Chondroitinase ABC can digest both CS and DS, while chondroitinase AC only target the CS structure (Nakano et al., 1996; Nakano and Ozimek., 1999). All samples and the standards (Δdi-0S, Δdi-4S and Δdi-6S) were derivatized by reductive amination using AMAC (Appendix D). The elution times of the disaccharides together with the *m/z* values are used to assign each disaccharide. Figure 4.4 shows the fluorescent AMAC derivatized unsaturated disaccharides Δdi-0S (Figure 4.4A), Δdi-4S (Figure 4.4B) and Δdi-6S (Figure 4.4C) eluted at retention times of 4.91, 5.71 and 5.68 min, respectively. The corresponding ESI-MS spectra indicate the respective disaccharides with unprotonated ion peaks at *m/z* 572.1 for Δdi-0S (Figure 4.4 A1), *m/z* 652.2 for Δdi-4S (Figure 4.4 B1) and Δdi-6S (Figure 4.4 C1). For the milk GAGs sample, two peaks were found after digestion with chondroitinase ABC (Figure 4.5B) with retention times at 4.89 and 5.69 min, with corresponding molecular masses of 572.1 *m/z* and 652.2 *m/z*, respectively (Figure 4.5 B1). Based on these retention times, both Δdi-0S and Δdi-6S units were present in the chondroitinase ABC digests of the milk GAGs. In addition, the DS control sample digested by chondroitinase ABC showed a similar peak profile, where two peaks were eluted at 4.89 and 5.69 min. This suggests the presence of DS in the milk GAGs. For the chondroitinase AC digest, the milk GAGs and control DS gave different peak profiles, where one single peak was eluted in the milk GAGs sample but no peak was found in the DS control sample. DS is not susceptible to chondroitinase AC thus no obvious peaks were found (data no shown).

The peak found in the milk GAGs chondroidnase-AC digest was eluted at a retention time 5.72 min (Figure 4.5 C) consistent with the retention time of Δdi-4S (Figure 4.4 B), indicating that the milk GAGs were susceptible to chondroitinase AC digestion. Coppa et al. (2011) had reported that the disaccharide composition of CS from bovine milk contained 60% of Δdi-4S, thus the CS structure could also be present in this milk GAGs mixture. These LC-MS results were consistent with the cellulose acetate electrophoresis results, implying the presence of a CS/DS structure in the milk oligosaccharides fraction.

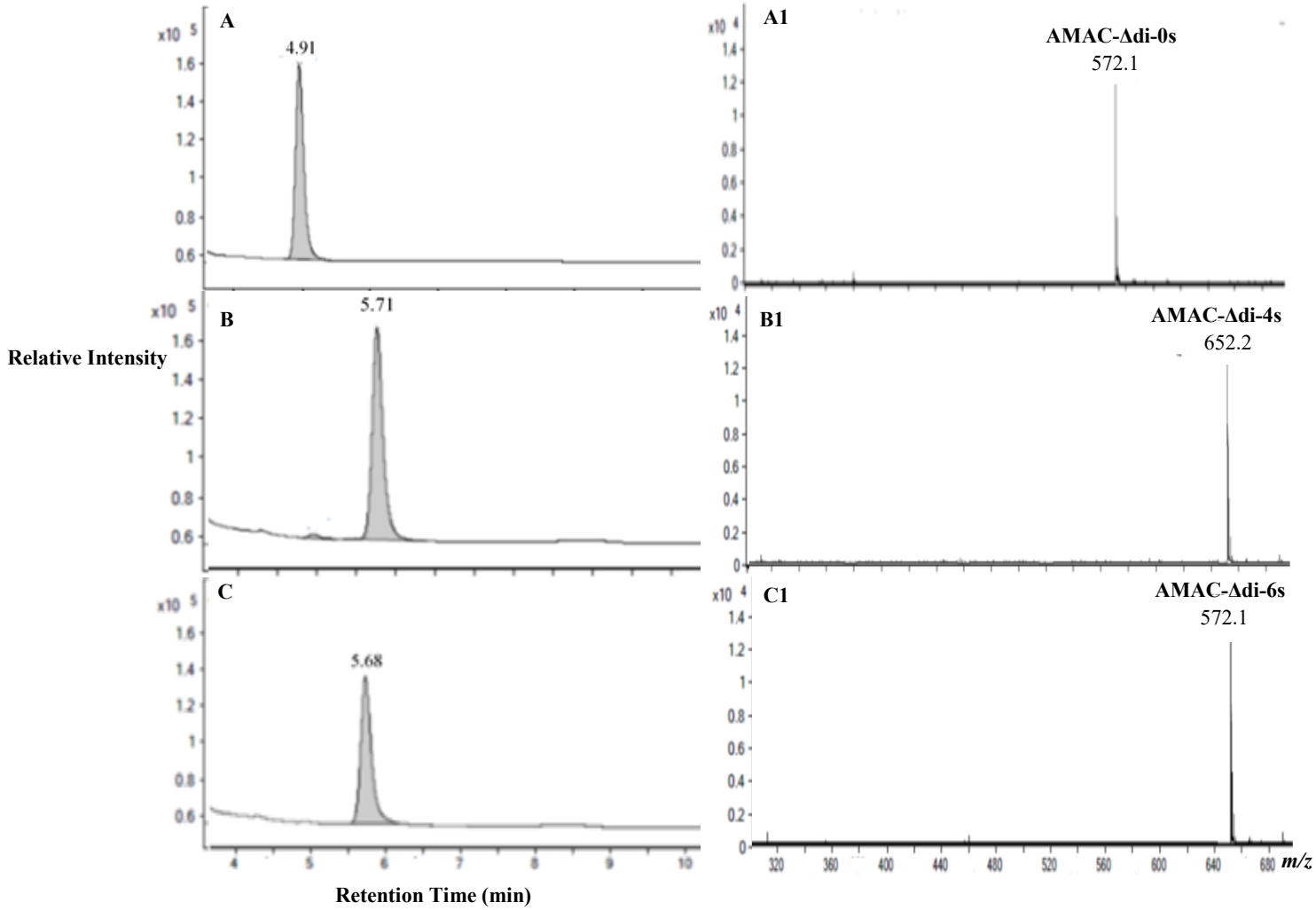

Figure 4. 4. HPLC chromatograms of AMAC-derivatized disaccharide standard solutions of (A) Δdi-0s (B) Δdi-4s, and (C) Δdi-6s monitored using fluorescence detection. The ESI-MS (-) of the respective disaccharides fluorescent derivatives of Δdi-0s, Δdi-4s and Δdi-6s are shown in A1, B1 and C1.

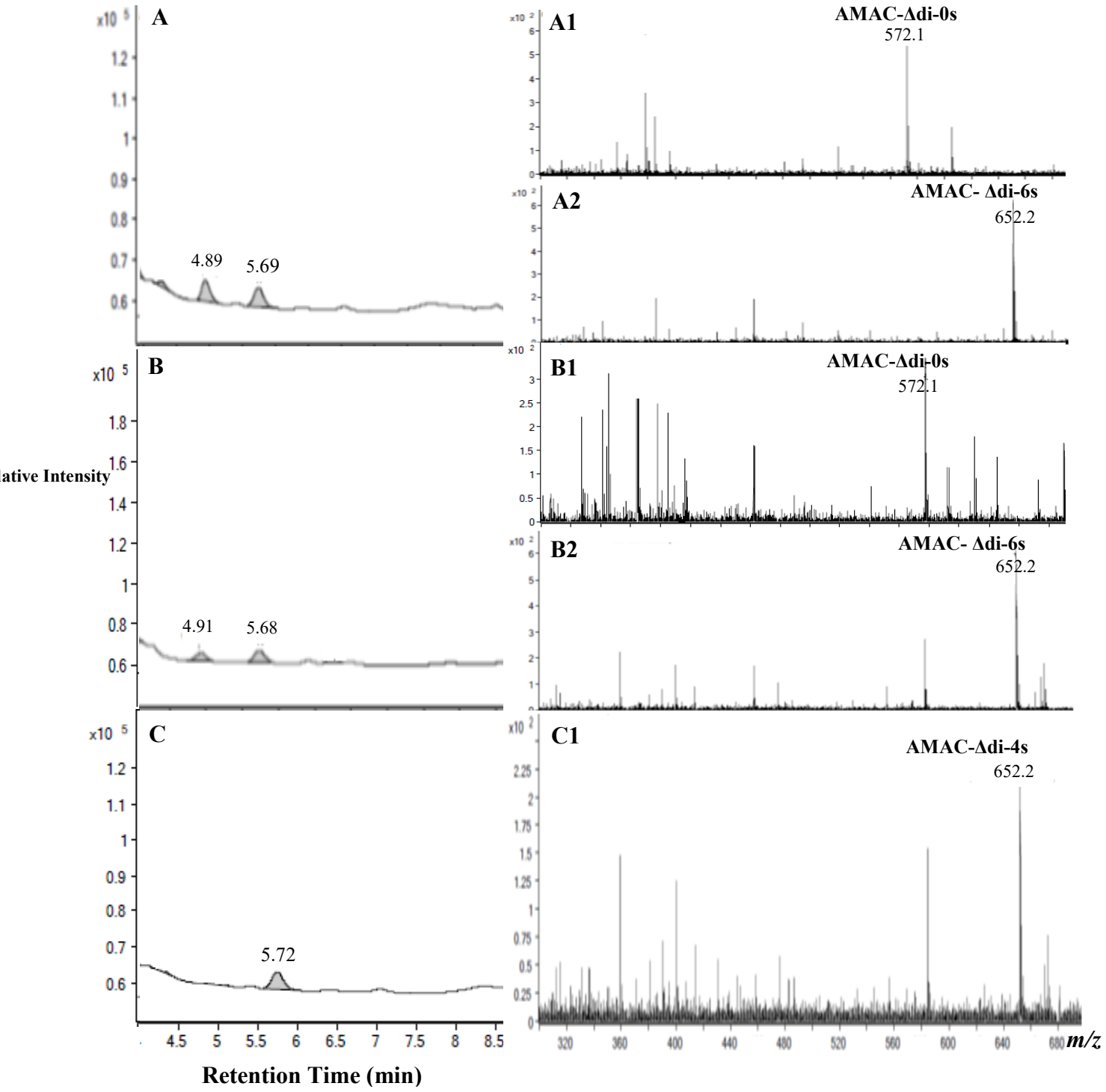

Figure 4. 5. HPLC chromatograms of AMAC-derivatized disaccharides from (A) dermatan sulfate standard digested with chondroitinase ABC, (B) bovine milk glycosaminoglycans digested with chondroitinase-ABC and (C) chondroitinase-AC. The ESI-MS (-) of the respective disaccharides are shown in A1 and 2; B1 and 2; and C1.

The CS/DS structure may directly interact with iron to improve its bioavailability as is found for Fe uptake by Caco-2 cells (Laparra et al. 2009). However, the CS/DS structure may be a minor part since the calculated milk GAGs quantity was the combination of 0.4 M and 2.0 M NaCl fractions (Figure 4.2). Nakano and Ozimek (1999) had indicated the presence of glycopeptides in their bovine milk GAG from a 0.4 M NaCl fraction. In this study, a relatively small amount of protein content was detected in the oligosaccharide fraction (0.91 ± 0.02 mg/mL). The minor protein portion in the milk oligosaccharide fraction was probably due to the peptides attached to GAGs. Thus, milk GAGs estimated from the oligosaccharides fraction could have been a mixture of CS, DS and glycopeptides.

4.3.4. Studies on iron uptake in a Caco-2 cell line

The low iron bioavailability of bovine milk sets up certain nutritional limitations for both infants and adults. The positive or negative effect of each milk component on iron absorption has been studied for many years; the results are both inconsistent and contradictory. In this study, ferritin synthesis was used to assess Fe uptake as an indirect measure of iron bioavailability. In the past several years, this measurement has been broadly accepted because iron can be stored as ferritin, and greater iron absorption increases ferritin synthesis in Caco-2 cells (García-Nebot et al., 2010; Glahn et al., 1998). The SBM was fractionated into four components: casein, whey, lactose and oligosaccharides (CS/DS structures). The supplemented CS-oligos that were produced by the enzymatic hydrolysis of CS from bovine trachea were between 1 and 10 kDa (chapter 3) and were negatively charged with sulfated groups. Many biological activities of GAGs depend on their molecular weight and chemical structure (Zivkovic and Barile. 2011; Coppa et al., 2013). Negatively charged moieties from these low molecular weight CS-oligos may confer many desired

properties for increasing iron absorption such as increased iron solubility by improving reducing power or forming soluble ligand with positively charged metal ions (chapter 3).

The Fe uptake studies were divided into three parts: 1) the effect of individual SBM components, 2) the effect of supplementation of CS-oligos into each milk component, and 3) the effect of modified SBM composition with and without supplementation of CS-oligos. These studies were designed to systematically compare the effect of each milk component on Fe uptake, particularly the effect of the milk oligosaccharide fraction since this has not been reported previously. More importantly, it was important to determine if the CS-oligos are a worthy candidate to enhance iron absorption as a supplement in dairy products.

4.3.4.1. Effect of the individual SBM components on Caco-2 cell Fe uptake

Ferritin/cell protein ratios were analyzed for the individual SBM fractions first without extrinsic iron added. The blank contained a ferritin level that represented the basal ferritin/cell ratio of the Caco-2 cells. SBM, whey and lactose indicated slightly greater amounts of ferritin formation, while casein and oligosaccharides showed ferritin levels that were not significantly different compared to the blank. No fractions showed ferritin levels greater than 20 ng/mg cell protein, which is considered relatively low and represented the effect of intrinsic iron from SBM. Each component had a limited influence on the baseline ferritin formation in Caco-2 cells. The addition of extrinsic iron significantly increased the ferritin formation in both blank and all SBM fractions with the exception of casein. This suggested that the intrinsic iron level in SBM is too small to study in order to get an appropriate level of ferritin formation by the Caco-2 cells. The extrinsic iron source was needed for this study and the addition of 20 μmol/L $FeSO_4$ was sufficient to see the different effects for each milk component.

With the addition of the extrinsic iron source, the greatest ferritin formation was found in SBM (56.9 ± 5.2 ng ferritin/mg cell protein), while casein showed the least ferritin formation (22.0 ± 2.3 ng ferritin/mg cell protein). The whey, lactose and oligosaccharides showed intermediate ferritin formation at 37.9 ± 7.9, 37.8 ± 5.6 and 36.8 ± 3.2 ng ferritin/mg cell protein, respectively. No statistical difference was found among the three fractions (Figure 4.6A).. These results suggest that each milk component affects the ferritin synthesis differently. The least ferritin formation from casein indicates its inhibitory effect on Fe uptake by Caco-2 cells. Casein protein from bovine milk has been proposed as the major inhibitory factor on iron absorption. This phosphoprotein can sequester Fe by clusters of phosphoserine to keep Fe soluble at the alkaline gut pH but preventing its absorption by duodenal mucosa Caco-2 cells (Jackson and Lee, 1992). The protein hydrolysis of whole casein may lessen this negative effect due to the release of the caseinophosphopeptides (CPP) that have a positive effect on Fe uptake. However, the effect of CPP on iron absorption is very dependent on the type of casein, the degree of hydrolysis and the concentration of active CPP (Kibangou et al., 2005). The two main caseins are α_s- and β-caseins; binding Fe to CPP of hydrolyzed β-casein generally improves its absorption but CPP from the hydrolysis of whole casein or fractions enriched with α_s-caseins have an inhibitory effect on iron absorption (Kibangou et al., 2005). The casein fraction used in this study was precipitated as whole casein, thus the casein peptides released after *in vitro* digestion did not improve the effect on ferritin synthesis. In addition, the SDS-PAGE analysis of casein protein before and after *in vitro* digestion (Appendix C) indicated that the hydrolysis was not fully complete, thus there were not enough active Fe-enhancing CPPs. On the other hand, calcium, the second best candidate for the inhibitory effect of iron absorption in SBM, mostly exists as colloidal calcium phosphate complexes with casein, and may be present in the casein fraction. The inhibitory mechanism of calcium on iron absorption can

be explained by calcium competing for common mucosal receptors with other cations like Fe or preventing the movement of iron thus inhibiting its uptake by mucosal cells (Etcheverry et al., 2004). Free calcium ions could be released during the *in vitro* digestion to interfere with the Fe uptake later by the Caco-2 cells.

Among the Fe added fractions, whey, lactose and oligosaccharides showed superior ferritin formation compared to the blank. These three milk components are considered to be soluble milk fractions or whey permeates after cheese making. Whey proteins/peptides are the major component that have been indicated as iron bioavailability enhancers, mainly based on their complexation with Fe during digestion and absorption (Caetano-Silva et al., 2017). The whey fraction in this study had intermediate values of iron bioavailability likely due to the presence of high- and low-molecular weight peptides as ligands. The larger molecular weight peptides may form stronger bonds to the Fe, enough to keep iron solubilized, but also enough to keep Fe from being donated to the Caco-2 cells, consequently impeding absorption (Sugiarto, Ye and Singh, 2009). A more recent study reported the effective Fe binding of whey peptides, which have a molecular weight of less than 5 kDa (Caetano-Silva et al., 2017), a size that is difficult to achieve by natural gastrointestinal digestion.

Lactose and oligosaccharides are the major carbohydrate fractions of milk but have often been neglected in studies of iron bioavailability in milk, despite having a similar iron bioavailability as whey protein hydrolysis (Figure 4.6A). Etcheverry, Miller and Glahn (2004) separated bovine milk whey into 10 kR (MW > 10kDa) and 10kF (MW < 10 kDa) fractions and reported that bovine milk whey 10 kF fractions elicited the greatest cell ferritin formation in a Caco-2 cell culture model. The study observed approximately a 3-fold less ferritin formation in the whey fraction compare to the results that reported by Etcheverry, Miller and Glahn (2004). One reason for such a result could

be that a whole whey fraction was used in this study. Previously, Etcheverry, Miller and Glahn (2004) reported that their whey 10 kF fraction had the least protein concentration, suggesting that this small molecular weight fraction was composed of carbohydrate, salt and vitamins. Lactose (MW ≤ 1 kDa) and oligosaccharides (1 kDa ≤ MW ≤ 10 kDa) were most likely in this whey 10 kF fraction and probably contributed to its superior iron bioavailability. In this study, when the three milk components were separated to test their effect on Fe uptake, their similar contribution to the ferritin level suggested that these soluble milk components all have a certain Fe-enhancing effect. In addition, bovine milk oligosaccharides were likely to have a positive effect on the iron status in animal and human models. For instance, two recent studies have reported a prebiotic effect on the gut microbiota by purified fructo- and galacto-oligosaccharides, which improved the iron status of both anemic rats and Kenyan infants (Zhang, Yung, Chung and Yeung., 2017; Paganini et al., 2017, respectively). Here, prebiotic metabolism by gut microbes produces short chain fatty acids, such as acetic, propionic and butyric, which then decrease the pH of the colon and presumably promote iron bioavailability (Wang and Gibson, 1993). To the best of knowledge, there are no studies that report how the milk oligosaccharides directly react with Fe and affect its uptake by Caco-2 cells. In this study, the milk oligosaccharides enriched from the commercial SBM showed a similar effect on Fe uptake compared to milk whey and lactose following *in vitro* digestion. This Fe-enhancing effect of milk oligosaccharides could be attributed to the CS/DS structures (Figure 4.5) associated with the milk GAGs, which contain negatively charged moieties that could increase iron solubility to improve its uptake by Caco-2 cells.

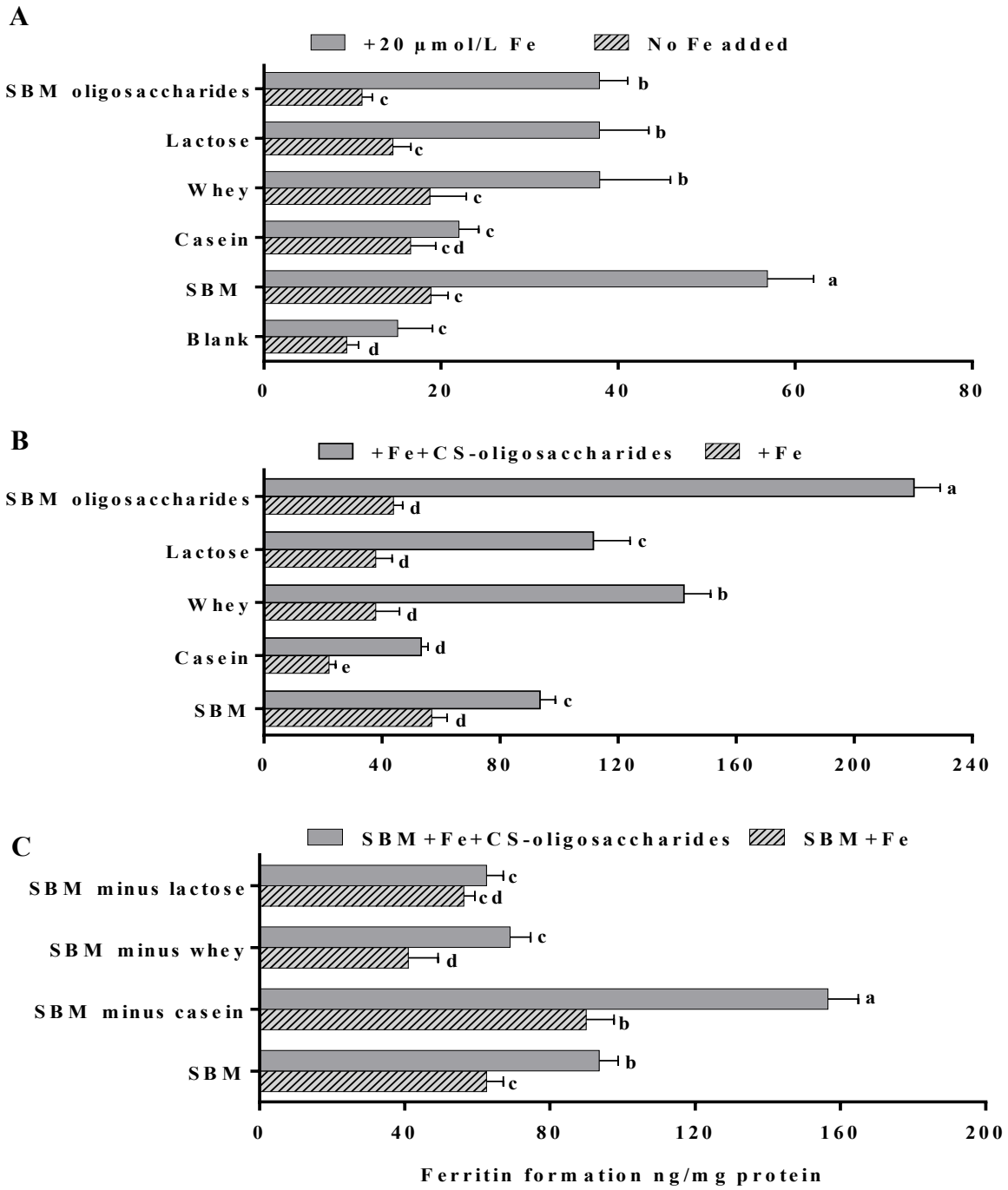

Figure 4. 6. Ferritin levels in Caco-2 cells exposed to the *in vitro* digested SBM fractions with and without $FeSO_4$ (A), the SBM fractions with and without CS-oligosaccharides supplement (B) and modified SBM fractions with and without CS-oligosaccharides supplement (C). All tested fractions from (B) and (C) were supplemented with iron (20 µmol/L as $FeSO_4$) and subjected to an *in vitro* digestion. Bars means ± standard deviation (n=3). The bars with different letters differ significantly at $P < 0.05$. SBM: skim bovine milk.

4.3.4.2. Effect of the CS-oligos supplementation with individual SBM fractions on Caco-2 cell Fe uptake

Figure 4.6 B shows the ferritin formation of SBM and each SBM component before and after supplementation of CS-oligos with extrinsic iron as determined in the previous section. After the addition of CS-oligos to the oligosaccharide fraction, the ferritin synthesis rates were 1.5-, 1.9-, 2.3- and 4.1-fold greater than that of the whey, lactose, SBM and casein fractions, respectively. It shows that the enhancing effect of CS-oligos on Fe uptake is most prominent in the milk oligosaccharides fraction and least with the casein fraction. The supplementation of CS-oligos likely increases the CS-like structures lacking in the bovine milk, thus leading to the greatest ferritin formation in Caco-2 cells. As reported in chapter 3, the CS-oligos exhibit superior effect on ferritin formation compared to DS-derived oligos.

In general, iron needs to be soluble in the lumen in order for to it be absorbed. If $FeSO_4$ is added as an extrinsic source (since ferrous iron is soluble), it should be better for ferritin synthesis. However, the absorption of ferrous iron still cannot be guaranteed since bovine milk contains iron absorption inhibitors (i.e., casein and calcium). Furthermore, ferrous iron can be converted to ferric iron in the human intestine. Ferric iron becomes insoluble at pH values greater than 3.0, such as the environment of the intestinal lumen. Therefore, the CS-oligos may improve iron absorption by either keeping the iron in its soluble form (Fe^{2+}; reducing effect) or by a chelation effect through forming a soluble complex with ferrous iron to protect iron from inhibitors or the alkaline conditions of the intestine. The superior Fe-enhancing effect of CS-oligos could be due to their ability to interact with iron in both ways. Firstly, the negatively charged groups such as sulfate and carboxylic groups have an iron-binding capacity capable of forming a soluble CS-oligos-iron complex. Also, the enzymatic hydrolysis of the glycosidic linkages generates more hydroxyl terminal groups, which in turn could increase the CS-oligos' reducing capacity to keep iron at a

low valence state and maintain its solubility (chapter 3). Moreover, with the addition of CS-oligos, the ferritin synthesis rate increased 2.4-fold in the casein fraction. Overall, it is likely that the capacity of CS-oligos to protect ferrous iron is able to overcome the inhibitory effect of casein.

The mechanism by which the CS may enhance iron to enter the enterocyte is still under debate; two hypotheses are being considered. One theory is that the formation of a CS- oligos-iron complex can maintain the solubility of iron to reach the absorption sites of the intestinal lumen. Next, the complexed iron is released prior to entering the enterocyte to be taken by DMT-1 a membrane divalent metal transporter involved in iron transport across intestinal cells. Alternatively, the CS-iron complex may be internalized across the intestinal brush border membrane through endocytosis due to cell reorganization of GAGs (Laparra et al., 2009). GAGs are metabolized in the acidic compartments of the enterocyte endosomal/lysosomal system. However, CS chains with a molecular mass greater than 10-25 kDa likely have a poor intestinal absorption (Baici et al., 1992). Therefore, since enzymatically hydrolyzed CS-oligos are low molecular weight compounds (≤ 10 kDa) with two functional groups, this may favour iron to enter the enterocytes and increase ferritin synthesis even with the presence of an Fe inhibitor such as casein in this study. In chapter 3, it was also found that CS-oligos showed a superior reducing power as well as a free radical scavenging activity. Thus, it is hypothesized that CS-oligos facilitate iron absorption by intestinal cells through two steps: firstly, CS-oligos maintain iron at its more soluble form of a low valence state to better promote transport via the normal DMT-1 receptor, and secondly, the CS-oligos-iron complex enters the intestinal cell through endocytosis.

4.3.4.3. Effect of the CS-oligosaccharides supplementation to modified SBM fractions on Caco-2 cell Fe uptake

The food matrix can have an important role in iron absorption to influence its bioavailability in many ways (Bovell-Benjamin, Viteri, Allen, 2000). In this study, it was important to determine if the enhancing effect of the CS-oligos on Fe uptake could still be pronounced in a milk mixture. Therefore, the effect of CS-oligos on Fe uptake was determined in the presence of SBM and modified SMB (Figure 4.6C). SBM is considered as a good food matrix model for studying iron absorption, because it contains both a dietary inhibitor (casein) and an enhancer (whey) of iron absorption. The ferritin synthesis rate was 1.5-fold greater in the SBM fraction after the addition of CS-oligos (Figure 4.6C). Moreover, the addition of CS-oligos in the SBM had a similar effect on Fe uptake as did the SBM minus whey fraction. This suggests that CS-oligos can overcome the inhibitory effect of casein on Fe uptake and its promoting effect on Fe uptake was not masked when applied to the milk digest. The SBM minus casein fraction elicited the greatest ferritin formation in Caco-2 cells among all modified SBM fractions after the addition of CS-oligos, suggesting that bovine milk casein acts as the main Fe uptake inhibitor. This agrees with the research by Etcheverry, Miller, and Glahn (2004). The greatest ferritin formation was observed in the SBM minus casein fraction after the addition of CS-oligos, implying that CS-oligos may be more effective for increasing dietary non-heme iron bioavailability in a dairy product without casein.

4.4. Conclusion

The evidence of this study shows how casein is the main Fe uptake inhibitor in milk after *in vitro* digestion. The whey fraction had an intermediate Fe uptake-enhancing effect when lactose-containing fractions were removed. This research is the first to report the effect of milk oligosaccharides on Fe uptake by Caco-2 cells, thus documenting an iron absorption promoting

effect by CS-oligos. It is proposed that this enhancement is probably due to the multiple Fe uptake-enhancing functional groups of CS-oligos. More importantly, CS-oligos were found to promote Fe uptake in an SBM food matrix model when the extrinsic iron was added. The inhibitory effect of certain milk components (i.e. casein) on dietary iron may be mitigated or reversed by the CS-oligos. This then suggests the possibility of supplementing CS-oligos into Fe-fortified bovine milk or infant formulas to enhance the Fe absorption. In addition, the presence of CS/DS structures found in the bovine milk oligosaccharide fraction suggests a possible prebiotic effect which should be further studied. Future animal and clinical studies should be conducted to provide more conclusive evidence of the enhancing effect of CS-oligos on Fe bioavailability. It is anticipated a range of practical applications could emerge from this study that would improve dietary non-heme iron bioavailability with dairy products; in Fe-fortified infant formula, to food aid rations, to sophisticated health energy bars, or even whey protein isolates that enhance performance in athletes.

Chapter 5. Chemical depolymerisation of chondroitin sulfate glycosaminoglycan improves both antioxidant activity and Fe uptake in a human intestinal Caco-2 cell model

5.1. Introduction

Chondroitin sulfate (CS) is a linear and negatively charged polysaccharide of repeating disaccharide units of glucuronic acid (GlcA) and *N*-acetylgalactosamine (GalNAc), usually sulfated at the C-4 and/or C-6 of the GalNAc units (Baici, Horler, Moser, Hofer, Fehr, and Wagenhauser, 1992). This sulfated polysaccharide is covalently attached to protein to form a proteoglycan and is abundant in the extracellular matrix of cartilaginous tissues (Nakano, Sunwoo, Li, Price, and Sim, 1996). Native CS extracted from mammalian tissues has been widely used in both the pharmaceutical and nutraceutical industries due to possessing antiarthritic, anti-inflammation and antioxidant bioactivities (Volpi, 2006). The negatively charged moieties on the CS polysaccharide chains likely confer the ability to improve iron (Fe) absorption by improving Fe solubility and reducing power, and likely form a soluble ligand with this positively charged metal (Laparra, Tako, Glahn and Miller, 2008). However, CS is a large molecular weight polymer (MW 20-100 kDa) with poor intestinal absorption, thus with restricted bioavailability (Baici et al., 1992). To improve bioavailability, it is essential to depolymerise these molecules into oligosaccharides so their therapeutic properties can be better manifested (Volpi et al., 1999; Toida, Sato, Sakamoto, Sakai, Hosoyama and Linhardt, 2009). Over the years, a number of approaches have been reported for the preparation of CS-derived oligosaccharides. The mildest approach is through controlled enzymatic depolymerisation (Toida et al., 2009). In chapter 3, compared to the intact CS polysaccharides, enzymatically depolymerised CS-oligosaccharides with a reduced molecular weight (≤ 10 kDa) had significantly better antioxidant capacity and also supported improved ferritin formation in human intestinal epithelia cells (Caco-2 cells) (chapter 3).

However, the enzymes required to depolymerise the CS are very expensive and would limit their application commercially. On the other hand, the strategy of using chemical depolymerisation of polysaccharides can be evaluated as a potential means of implementing such a technology into commercial context. Similarly, to other polysaccharides, CS can be systematically degraded by acid or alkaline hydrolysis with increasing time and temperature (Tokita and Okamoto, 1995; Volpi et al., 1999; Jandik, Kruep, Cartier and Linhardt, 1996).

Acid hydrolysis is a conventional, economical and simple approach for polysaccharide depolymerisation (Lu, Ai, Guo, Fu, Cao and Song, 2017). For acid-catalyzed hydrolysis, depolymerisation and desulfation occur together, which can impact the Fe-uptake enhancing effect of the depolymerised CS products, since the negatively charged sulfate moieties may be responsible for the formation of soluble ligands with Fe. On the other hand, similar to other polysaccharides, CS can also go through the β-elimination of the substituent attached at O-4 under alkaline conditions without substantial loss of sulfate groups (Volpi et al., 1999). Thus, alkaline β-elimination can also be a strategy for the depolymerisation of CS, where the process selectively splits the 1→4 glycosidic linkage to form $\Delta^{4,5}$ unsaturated uronic acid residues at the non-reducing end of resulting fragments (Inoue and Nagasawa, 1986; Hirsh and Levine, 1992; Jandik et al., 1996). At the same time, CS also contains GalNAc at the other end of the disaccharide unit, which can go through deacetylation during alkaline hydrolysis. Chitosan, the other *N*-acetyl-amino sugar containing oligosaccharides, is obtained from chitin by deacetylation under alkaline hydrolysis. The degree of deacetylation (DD) of chitin is an important parameter that determines the physiochemical and biological properties of the resulting chitosan (Yuan, Chesnutt, Haggard and Dumgardner, 2011). However, the sulfated polysaccharides are reported to be more resistant toward direct β-eliminative depolymerisation in alkaline solutions (Gao et al., 2015). The acid-

derived CS oligosaccharides could be used as a substrate for the non-enzymatic browning reaction accelerated by alkaline conditions. Thus, in this study, a set of combined treatments using acid and alkaline conditions was evaluated.

The biological activities of CS depend on their molecular weights and degree of sulfation. Therefore, the objectives of this study were: (1) to evaluate the physico-chemical properties of depolymerised CS products under both acid, alkaline and combined (acid and alkaline) treatments; (2) To compare the antioxidant capacity of these resulting CS-hydrolysates, determined by measuring radical scavenging activity, ferric reducing and iron chelating activities; (3) To determine the effect of CS-fragments derived from chemical hydrolysis on Fe uptake by Caco-2 cells. The hypothesis here is that the chemical hydrolysis effects on CS structure influence its antioxidant capacities as well as its capacity of Fe uptake by Caco-2 cell.

5.2. Materials and Methods

5.2.1. Materials

Chondroitin sulfate sodium salt (70% chondroitin sulfate A; 30% chondroitin sulfate C) from bovine trachea, bovine testicular hyaluronidase (EC3.2.1.35), *N*-acetyl-D-galactosamine, galactosamine, disaccharide standards (Δdi-0S, Δdi-4S and Δdi-6S), pancreatic enzymes from porcine pancreas, pepsin from porcine gastric mucosa and bile extract and *o*-phthaldialdehyde (OPA) were obtained from Sigma–Aldrich (Mississauga, ON, Canada). Hydrochloric acid, sodium hydroxide, anhydrous sodium phosphate dibasic, anhydrous sodium carbonate, dibasic, and sodium azide were from Fisher Scientific (Ottawa, ON, Canada). Chelex-100 was from Bio-Rad Laboratories (Hercules, CA, USA). Diethylaminoethyl (DEAE) cellulose anion exchanger (DEAE Sephacel) was obtained from GE Healthcare, Bio-Science (Mississauga, ON, Canada). 2-Aminoacridone (AMAC, >98%), glacial acetic acid, dimethyl-sulfoxide (DMSO, 99.9%), and

sodium cyanoborohydride (95%) and all other reagents, of the analytical grade, were from Sigma-Aldrich. All solutions were prepared with Milli-Q purified deionized (DI) water (17 MΩ·cm, Millipore, Bedford, MA, USA).

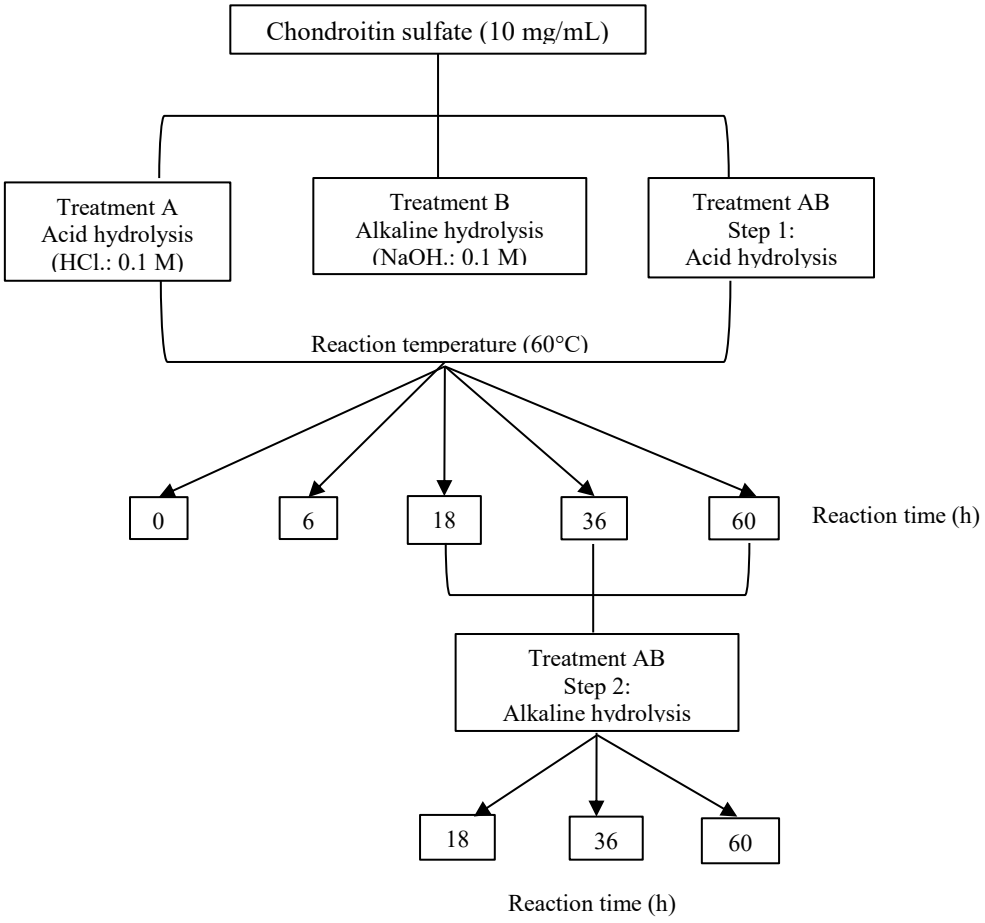

Figure 5. 1. Experimental variables for chemical depolymerisation of chondroitin sulfate with different treatments. (Treatment with acid: A, Treatment with alkaline: B, Treatment with acid followed by alkaline: AB)

5.2.2. Degradation of CS under acidic and alkaline conditions

According to the method reported by Volpi et al. (1999), CS (10 mg/mL) was degraded under both acidic and alkaline conditions, performed in 0.1 M HCl (pH 1.5-2.0) and 0.1 M NaOH (pH

11.5-12.0) solutions, respectively. These solutions were incubated in water baths at 60°C. Aliquots were removed at various times (from 0 to 60 h) and neutralized with an equal volume of either 0.1 M NaOH or 0.1 M HCl, followed by an equal volume of 50 mM sodium phosphate buffer (pH 7.0). A control sample was also prepared by CS (10 mg/mL) in 10 mM sodium phosphate pH 7.0. Based on the preliminary data, a combined (acid and alkaline) treatment was also used in this study (Figure 5.1): CS was pre-treated with an acid solution, aliquots were removed at 18, 36 and 60 h and then adjusted by 0.1 M NaOH to alkaline condition at pH 11.5-12.0. Then incubated in water bath at 60°C for another 18, 36 and 60 h. Aliquots were removed and neutralized with an equal volume of 0.1 M HCl, followed by an equal volume of 50 mM sodium phosphate buffer. All samples were desalted by ultrafiltration through 650 Da molecular weight cut off membrane for 72 h at 4°C. Samples were then freeze dried and stored at -20°C for further analysis.

5.2.3. Determination of reducing end groups of chemically hydrolyzed CS

Samples from acidic, alkaline and combined (acid and alkaline) treatments were analyzed to determine the moles of reducing sugar present at each time point. The assay was performed as reported by Jandik et al. (1996). Freeze dried samples were dissolved in DI water and centrifuged using 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 to remove any precipitation. A standard curve was prepared using *N*-acetyl-D-galactosamine to calculate the nanomoles of reducing sugar present in the hydrolyzed CS samples.

5.2.4. Cellulose acetate electrophoresis of the hydrolyzed CS from different treatments

Electrophoresis on cellulose acetate strips was carried out for samples (1.0 mg/mL) incubated under buffer, acidic, alkaline and combined (acid and alkaline) conditions at each time point

according to Hata and Nagai (1971). After electrophoresis, each strip was stained with 0.1% (w/v) Alcian blue 8GX in 0.1% acetic acid and washed extensively with deionized water.

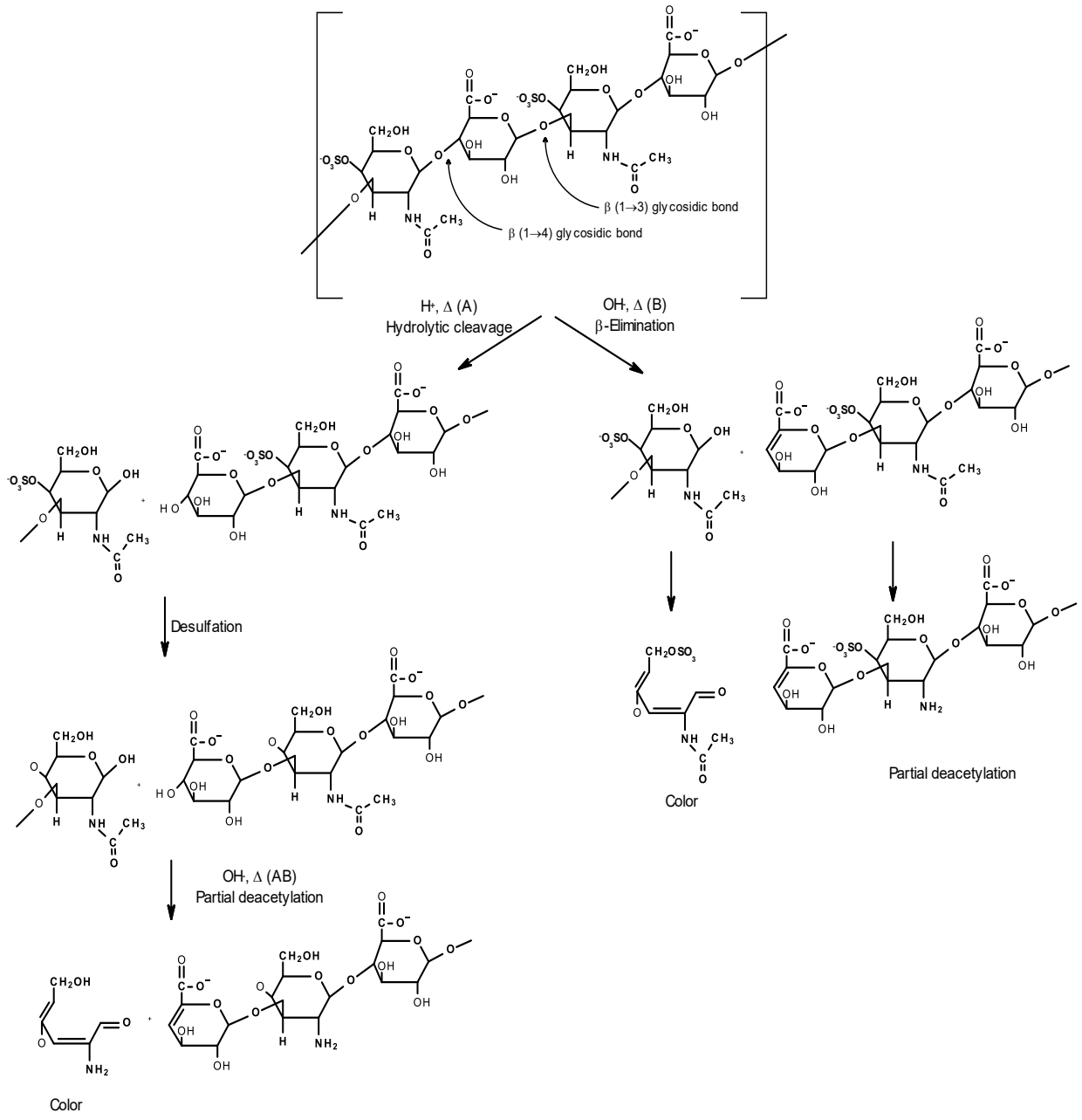

Figure 5. 2. Mechanisms of the hydrolysis of CS under acidic (A) and alkaline (B) conditions. Possible effect on the functional groups (sulfate and *N*-acetyl groups) of CS under acidic and alkaline conditions.

5.2.5. Determination of degree of deacetylation and sulfate group of the chemically hydrolyzed CS

A colorimetric quantification of the amount of primary amine formed during the chemical hydrolysis of CS was determined spectrophotometrically after derivatization with o-phthalaldehyde (OPA) and a thiol-dithiothreitol (DTT) according to the method by Larionova, Zuaerova, Gguranda, Pechyonkin and Balabushevich (2009) with some modifications. Galactosamine was dissolved in 0.2 M borate buffer, pH 8.9. to prepare a 0.1 mg/mL standard solution. A series of 0.01, 0.02, 0.04, 0.06, 0.08 and 0.1 mg/mL standard solutions was prepared from the 0.1 mg/mL standard solution. The hydrolyzed CS samples were dissolved in 0.2 M borate buffer, pH 8.9, to a final concentration of 1 mg/mL. The OPA reagent solution was freshly prepared immediately prior to the colorimetric assay by adding 200 μL of each of the ethanol solutions: 0.11 M o-phthalaldehyde and 0.071 M DDT to 5.0 mL of 0.2 M borate buffer, pH 8.9. Standards and samples (50 μL) were mixed well with 2 mL OPA reagent and then incubated for 2 min at 25°C. The absorption of each solution at 340 nm was determined against 0.2 M borate buffer as a blank in a Spectramax M3 multi-mode microplate reader (Molecular Devices, Sunnyvale, CA, USA) with a 1-cm quartz cuvette. According to the standard curve, the concentration of primary amine group formed in the hydrolyzed CS sample can be calculated. The amount of *N*-acetylagalactosamine in the CS was determined by the method described by Wu and Zivanovic (2008). *N*-Acetylglucosamine was used as the standard and the absorption of standard and CS at 199 nm was determined using DI water as reference. Based on the concentration of primary amine groups formed during the chemical hydrolysis of CS and the concentration of the *N*-acetylgalacotosamine initially present in the CS, the degree of deacetylation was calculated as below:

$$DD\ \% = C_{GalN} / C_{GalNAc} \times 100$$

where C_{GalN} is the galactosamine concentration of the hydrolyzed CS and C_{GalNAc} is *N*-acetylgalactosamine concentration of the CS.

The sulfate content in the hydrolyzed CS samples was determined with the sodium rhodizohate method according to Terho and Hartiala (1970).

5.2.6. Determination of AMAC-labeled Δ-disaccharides by RP-UPLC

Derivatization of Δ-disaccharide standards or the chemically hydrolyzed CS with AMAC was performed as previously described by Volpi, Galeotti, Yang and Linhardt (2014) with minor modifications. The freeze-dried sample was reconstituted with 5 μL of a 0.1 M AMAC solution in glacial acetic acid/DMSO (3:17, v/v) and 5 μL of a freshly prepared solution of 1.0 M sodium cyanoborohydride in water. Then, the mixtures were centrifuged in a microfuge at 11,000 X g for 3 min. Derivatization was performed by incubating at 45˚C for 4 h. Finally, 190 μL of 50% v/v DMSO were added to the samples. UPLC separation was performed on an Ascentis Express ES-C18 column (150 × 4.6 mm, 2.7 μm 221 particles) (Sigma-Aldrich, MO, USA). Eluent A was 60 mM ammonium acetate pH 5.6, and eluent B was acetonitrile. The samples were injected, and a gradient from 5 to 10% eluent B in 5 min, from 10 to 20% B in 50 min, and from 20 to 50% B in 10 min, at a flow rate of 0.5 mL/min, was used. Detection of the CS disaccharides achieved by fluorescence detection exiting at 442 nm and detecting at 520 nm.

5.2.7. Determination of antioxidant capacity

5.2.7.1. 2,2-Diphenyl-1-picrylhydazyl (DPPH) radical scavenging activity

The DPPH possesses an unpaired electron and exhibits a stable violet color in methanol solution (peak absorbance at 517 nm), is commonly used as a reagent for evaluation of the free radical scavenging activity of antioxidants (Li et al. 2012). DPPH radical scavenging effect is based on the reduction of DPPH in the presence of a hydrogen-donating reagent due to the

formation of the non-radical form (DPPH-H) in the reaction. In this method, a microplate assay method was used according to Li et al. (2012). Methanolic DPPH (200 µM; 100 µL) was mixed with 100 µL aliquot of sample at final concentration of 1.0 mg/mL into a 96-well microplate. A sample blank was prepared accordingly to minimized the color interference from the sample. The plate was gently mixed and incubated in the dark for 30 min at room temperature. The change in color (from deep violet to light yellow) was measured at 517 nm on a UV visible light spectrophotometer (SpectraMax M3, Molecular Devices, Sunnyvale, CA, USA). Ascorbic acid was used as an antioxidant standard and positive control. DPPH radical scavenging activity was expressed as the percentage inhibition and was calculated using the following formula:

DPPH radical scavenging activity (%) = [(*Abs*control – *Abs*sample) / (*Abs*control)] ×100

*Abs*control: absorbance of DPPH radical + methanol

*Abs*sample/positive control: (absorbance DPPH radical + sample or ascorbic acid)- absorbance of sample blank (sample dissolve in methanol)

5.2.7.2. Ferric reducing activity

The ferric reductase assay was performed as described previously (chapter 3). Briefly, a total volume of 0.2 mL of reaction mixture contained sample, ferric chloride (50 µM) and ferrozine (5.0 mM) with final sample concentration of 1.0 mg/mL and distributed into a microplate. Sample blank was also prepared by mixing sample and ferric chloride to correct the color interference result from the chemical hydrolysis process. The plate was shaken vigorously and incubated at room temperature for 30 min. Ascorbic acid was used as a positive control. The absorbencies of the mixtures were measured at 562 nm using Spectramax 3 microplate reader. Formation of ferrous

iron was calculated by using standard curve prepared with ammonium ferrous sulfate and the specific activity expressed as ferrous iron formed (μM)/ferric iron added (μM).

5.2.7.3. Iron chelating activity

The chelating effect of acidic, alkaline and combined (acidic and alkaline) hydrolyzed CS on ferrous ion was assayed according to the method described by Decker and Welch (1990) with modifications. The reaction mixture, containing sample, ferrous chloride (0.5 mM) and ferrozine (5.0 mM), was adjusted to a total volume of 0.2 mL with final sample concentation of 1.0 mg/mL. Sample blank was prepared by mixing sample and ferrous chloride. The plate was shaken vigorously and incubated at room temperature for 30 min. The absorbance of the mixture was measured at 562 nm against a blank. The blank was prepared in the same manner except that DI water was used instead of sample. Ethylenediaminetetra-acetic acid (EDTA) was used as a positive control. The percentage inhibition of ferrozine-$Fe2^{+}$complex was calculated using the following formula:

Iron-chelating activity (%) = [(*Abs*control – *Abs*sample) / *Abs*control] × 100

*Abs*control: absorbance of the blank

*Abs*sample/positive control: absorbance of the sample or EDTA-absorbance of sample blank

5.2.8. Fe uptake analysis by Caco-2 cell model

5.2.8.1.Caco-2 cell cultures

The Caco-2 cells were purchased from the American Type Culture Collection (Rockville, MD., USA) and used in all experiments described in this study between passages 25-30. Stock cultures were maintained in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Grand Island, NY, USA), pH 7.4, supplemented with 20% (v/v) fetal bovine serum (GIBCO), 5% HEPES and 1%

antibiotic antimycotic solution (GIBCO). The cell culture procedure is referred to section 3.2.9. in chapter 3.

Trans-epithelial electrical resistance (TEER) was utilized, using an EVOM2 Epithelial Voltohmmeter (World Precision Instruments, Hitchin, UK), before the iron uptake experiment to confirm the time to confluence and the integrity of the cell monolayers. The MTT (3-[4,5-dimethylthiazol-2-yl]-2,3-diphenyl tetrazolium bromide) cell proliferation assays (American Type Culture Collection, Manassas, VA., USA) were performed with all the samples at a range of concentration (0 to 2.0 mg/mL) with a 0.1 mg/mL interval (data not shown).

5.2.8.2. In vitro digestion of chemical hydrolyzed CS and iron uptake into Caco-2 cell

Stimulated gastrointestinal digestion and iron uptake into Caco-3 cell from the mixture of extrinsic $FeCl_3$ were performed. The detailed procedure was as previously described (Glahn, Lee, Yeung, Goldman, and Miller, 1998). Briefly, all samples were mixed with 50 μmol/L $FeCl_3$ at final concentration of 1.0 mg/mL (based on the MTT cell proliferation assay). A mixture containing $FeCl_3$ (50 μmol/L)/ ascorbic acid (AA) mixture (molar ratio for Fe/AA: 1/20) was used as the positive control. $FeCl_3$ and unhydrolyzed CS were used as the negative controls. The baseline cell ferritin was measured as the blank. Peptic and intestinal digestions were conducted on a rocking platform shaker and placed in an incubator at 37°C. After the gastric step (pepsin/pH 2.0/1h), the intestinal step (pancreatine-bile/pH 7/2 h) was carried out by adding 1 mL of the digest (final concentration of 1.0 mg/mL) into the apical chamber of the two-chamber system in 12-well permeable support plates and incubated at 37°C. MEM (1.0 mL) was added to the basolateral chamber. After 2 h, the apical and basolateral media were removed and collected into separate tubes. The apical surface of the cells was washed twice with 1.0 mL of PBS (pH 7.4) (10 mM phosphate buffer, 2.7 mM KCl, 137 mM NaCl) and an additional 1.0 mL of MEM were added.

The cells were next incubated for 22 h, washed five times with PBS, and then harvested in 2 mL of PBS and sonicated for 10 min at 4°C for protein and ferritin analysis. Ferritin content was measured with 1-stage sandwich immunoradiometric assay (Feriron II Ferritin Assay, Ramco laboratories, Houston, TX, USA). Cell protein was assessed using BCA protein assay kit (PierceTM , Fisher Scientific, Toronto, ON, Canada). The ratio of ferritin/total cell protein expressed as nanograms of ferritin per milligram of protein was used to estimate the content of ferritin formed in Caco-2 cells.

5.2.9. Statistical analysis

Each treatment was performed in triplicate. The experimental data were subjected to the one-way analysis of variance (ANOVA) and means were separated using Tukey test with $p < 0.05$.

5.3. Results and discussion

5.3.1. Effect of acid, alkaline and combined (acid and alkaline) hydrolysis on Chondroitin sulfate

The mechanism of hydrolysis of glucosides generally involves two basic pathways: one is through hydrolytic cleavage and the other through β-eliminative cleavage. The glycosaminoglycan-degrading enzymes could act either by elimination or hydrolysis on the depolymerisation of the polysaccharides without obvious impact on the structure of the resulting oligosaccharides (Ernst, Langer, Cooney and Sasusekharan, 1995). Both acid (hydrolytic cleavage) or alkaline (β-eliminative cleavage) treatments can cause desulfation and deacetylation as side effects (Figure 5.2). β-elimination under alkaline conditions cleave CS polysaccharides into oligosaccharides containing a $\Delta^{4,5}$ unsaturated uronic acid residue at the nonreducing end, which is similar to the action of chondroitin lyases (Inoue and Nagasawa, 1986; Hirsh and Levine, 1992; Jandik et al., 1996). However, Volpi et al. (1999) noted that alkaline treatment at 60°C was unable

to substantially degrade CS polysaccharides even after prolonged incubation (up to 960 h), while a complete CS depolymerisation occurred after 96 h of acidic treatment. Tokita and Okamoto (1995) also proposed that the cleavage of the glycosidic bond usually takes one step under acidic conditions, whereas under alkaline conditions it may involve two steps.

In this study, the chemical depolymerisation of CS under acidic, alkaline and combined (acid and alkaline) treatments was monitored at each time point using cellulose acetate electrophoresis visualized by staining with Alcian Blue dye (Figure 5.3). The position of the depolymerised CS band depends on its chain length and the number of sulfate groups on these hydrolyzed chains (Jandik et al., 1996). The acid treatment of CS resulted in a decreased stain intensity of the electrophoretic bands as a function of time, suggesting a reduction of CS-chain length and desulfation of the polysaccharides (Figure 5.3A). No obvious changes were found for the band staining intensity for alkaline treated CS during the incubation times (Figure 5.3B). This means there was no appreciable desulfation and β-elimination of polysaccharides under basic conditions after 60 h. For the combined treatment, acid hydrolysis was stopped at 36 h and then incubated under alkaline conditions for 18, 36, and 60 h. The electrophoresis of the combined (acid and alkaline) treated CS at 18 h (Figure 5.3C: lane 1) had similar intensities as did the acid treated CS at 60 h, suggesting that depolymerisation and desulfation had taken place, with total degradation occurring at 60 h (Figure 5.3C: lane 3).

The CS chain contains both a reducing and nonreducing end, and each time the chain is depolymerised, a new reducing and nonreducing end should be formed (Mopper and Gindler, 1973). The reducing ends were measured as a function of time in the chemically treated samples. These results demonstrate a significant rise in the number of reducing ends in the acid-treated sample, while no such increase was observed in the sample treated with alkaline (Figure 5.4A).

The increased amount of reducing end groups in the acid-treated CS indicate more hydroxy groups (-OH) formed due to hydrolytic cleavage, which agreed with the electrophoretic band pattern (Figure 5.3A). A possible reason for not achieving the same effects in the alkaline condition is likely due to the limited β-elimination cleavages on CS under alkaline condition; Figure 5.3B (cellulose acetate electrophoresis results) supports this conclusion. The results also agree with Volpi et al. (1999). The combined (acid and alkaline) treated sample showed a greater amount of reducing groups formed than for the alkaline treated sample at all three chosen time points (18, 36 and 60 h). This suggests that alkaline hydrolysis of CS chains may be more efficient after a certain level of hydrolytic cleavages have been achieved by the acidic pre-treatment.

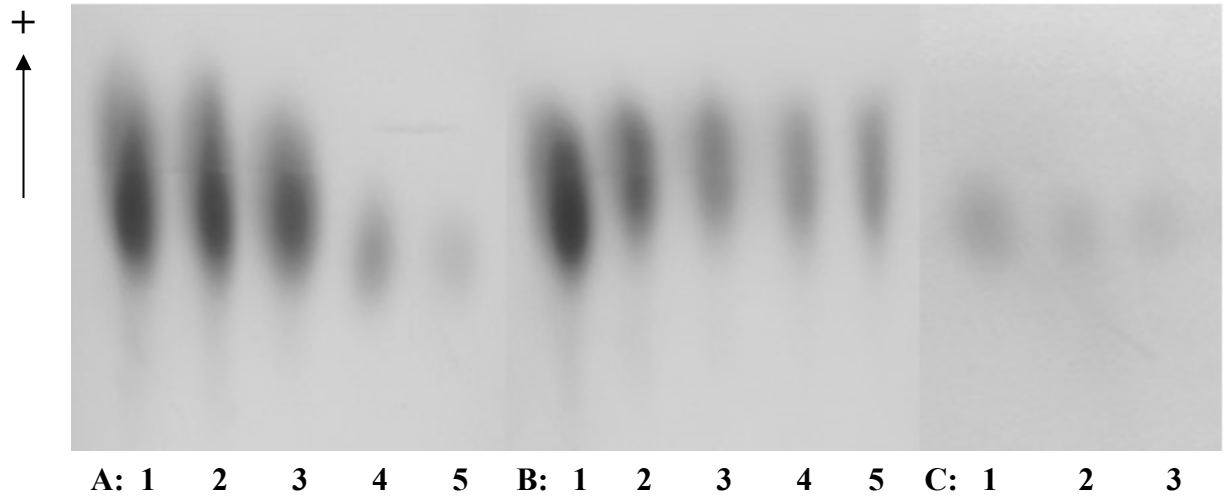

Figure 5. 3. Cellulose acetate electrophoresis of the acidic (A), alkaline (B) and combined (C) hydrolyzed CS at 60 °C as a function of time: A lane 1-5: acidic hydrolyzed CS at incubation time 0, 6, 18, 36, 60 h; B lane 1-5: alkaline hydrolyzed CS at incubation time 0, 6, 18,63, 60 h; C lane 1-3: acidic (36h) then alkaline hydrolyzed CS at 18, 36, 60 h. The arrow indicates the direction of the mobility.

5.3.2. Degree of deacetylation of hydrolyzed CS

The basic repeating disaccharide block that formed the backbone of CS consists of glucuronic acid (GlcA) and *N*-acetylgalactosamine (GalNAc). Tokita and Okamoto (1995) suggested that the

acidic hydrolysis affects the glucuronic acid moiety, whereas the alkaline hydrolysis attacks the *N*-acetylgalacosamine unit. Similar to the alkali treated chitosan, the GalNAc deacetylation could take place during alkaline hydrolysis of CS. The primary amino group would be formed on the GalNAc unit when the acetyl group is being removed (Figure 5.2B). The degree of deacetylation (DD) is determined using the quantitative colorimetric change of depolymerised CS bearing primary amino group based on the derivatization of their amino groups with a mixture of *o*-phthaladehyde and a thiol (Larionova et al., 2008). In this study, the DD of the chemically treated CS was determined and presented in Figure 5.4B. Examination of the formation of primary amino groups as a function of time showed a marked increase for alkaline treated samples. By the end of the incubation time, up to 92.1 % of DD was achieved by alkaline treatment. No such increase in DD was observed in the acid-treated samples, and only ~21% of DD was found after 60 h in acid incubation. The combined (acid and alkaline) treated samples showed an intermediate DD between acid and alkaline depolymerisation. The deacetylation patterns in acid and alkaline hydrolyzation of CS found in this study agree with the study reported by Liu, Linhardt and Zhang (2014). They found that the final concentration of the free acetate anion detected in the alkaline-treated heparin solution was about 4-5 times greater than that observed in the acid solution, which suggests that the acidic solution insufficiently hydrolyses the GAG *N*-acetyl groups. The alkaline deacetylation toward GAG molecules could involve the nucleophilic attack of OH^- on the carbonyl of the *N*-acetyl groups (Liu, Linhardt and Zhang, 2014). The DD of the combined (acid and alkaline) sample can likely be attributed to the second step alkaline incubation.

5.3.3. Changes in the sulfate content of hydrolyzed CS

Sulfate groups attached on the CS polysaccharide backbone is one of the most important features of these bioactive polyanion molecules, due to their ability to interact with proteins,

cellular components and metal ions such as Fe, conferring pharmacological and health promoting properties. Sulfate groups were found in a decreasing trend in all three chemically-hydrolyzed samples as a function of time (Figure 5.4C). Acidic and combined (acid and alkaline) depolymerised CS lost more sulfate groups as compared to the alkaline treatments. This intense reduction of CS's sulfation under acidic and combined (acid and alkaline) conditions, correlated with the mobility of CS treatments on stained samples after cellulose acetate electrophoresis. Visually, no or a small loss of sulfated groups in the alkaline treated samples corresponded to the large number of sulfated groups remaining in alkaline treated CS (Figure 5.4C). These results confirm that the sulfate groups in CS are more sensitive to acid than to alkaline. The likely mechanism involving the protonation of the oxygen of the sulfate group by acid following hydrolysis was discussed in several stability studies of heparin and CS (Jandik et al., 1996; Volpi et al., 1999; Liu, Linhardt and Zhang, 2014). The reduced molecular weight with different levels of loss of sulfate groups in three chemically treated samples had also been suggested by the detection of unsulfated disaccharides units by RP-UHPLC (Figure. 5.5). The acidic (60 h) and combined (acid 36 h and alkaline 60 h) treated CS were tagged by AMAC and both had a peak representing the unsulfated disaccharides unit (AMAC-Δdi-0S) at a retention time of 40.1 min, corresponding to the retention time of the AMAC-Δdi-0S standard. This implies that both depolymerisation and desulfation had taken place. However, no peaks were observed in the alkaline treated sample suggesting that some degradation of the CS polysaccharide backbone chain and no or small loss of sulfate groups. Liu, Linhardt and Zhang (2014) also found that the level of released sulfate anions from heparin in the alkaline condition was less than that for the sample incubated under acidic conditions. The double peaks observed in the standard and sample might be due to alpha or beta isomers of the disaccharides units.

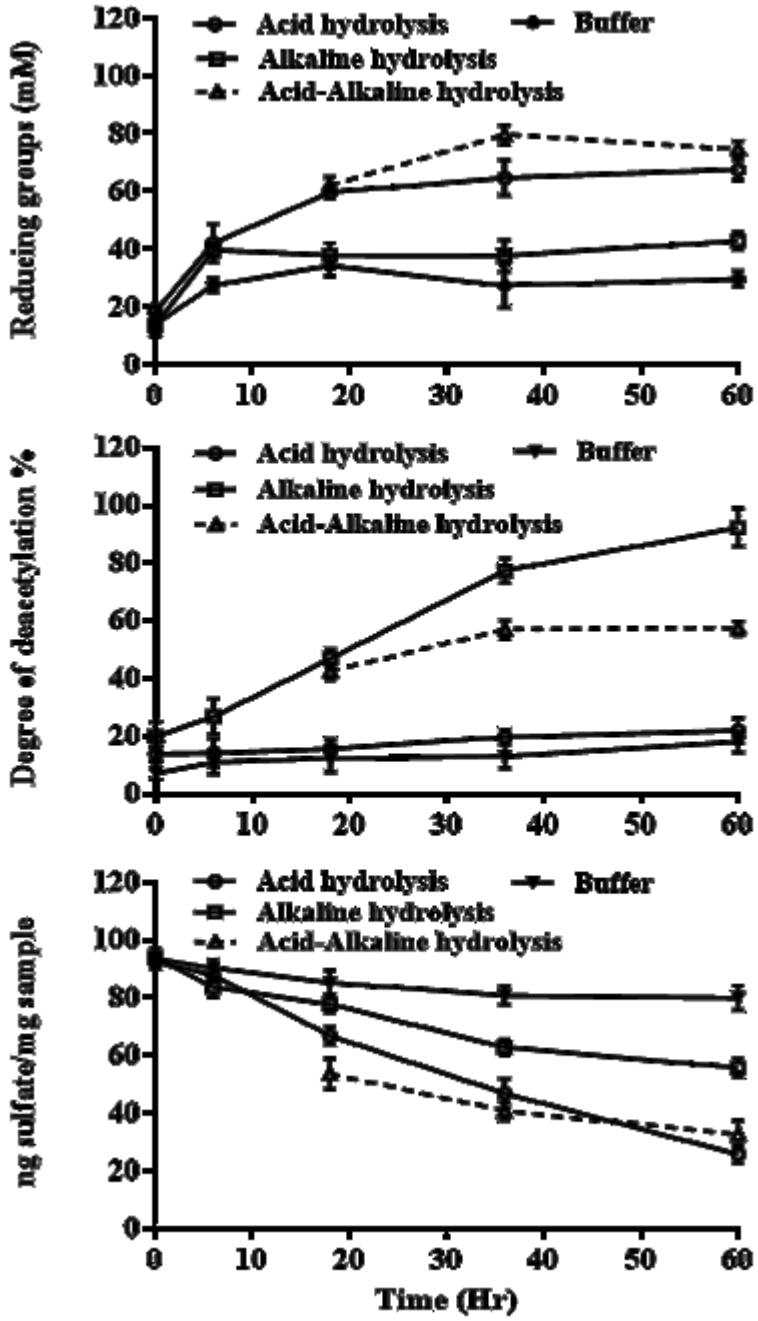

Figure 5. 4. The degradation of CS in acid, alkaline and combined (acid and alkaline) conditions were monitored by the (A) reducing end groups formed, (B) degree of deacetylation and (C) sulfate content (C) as a function of time (h) at 60 °C.

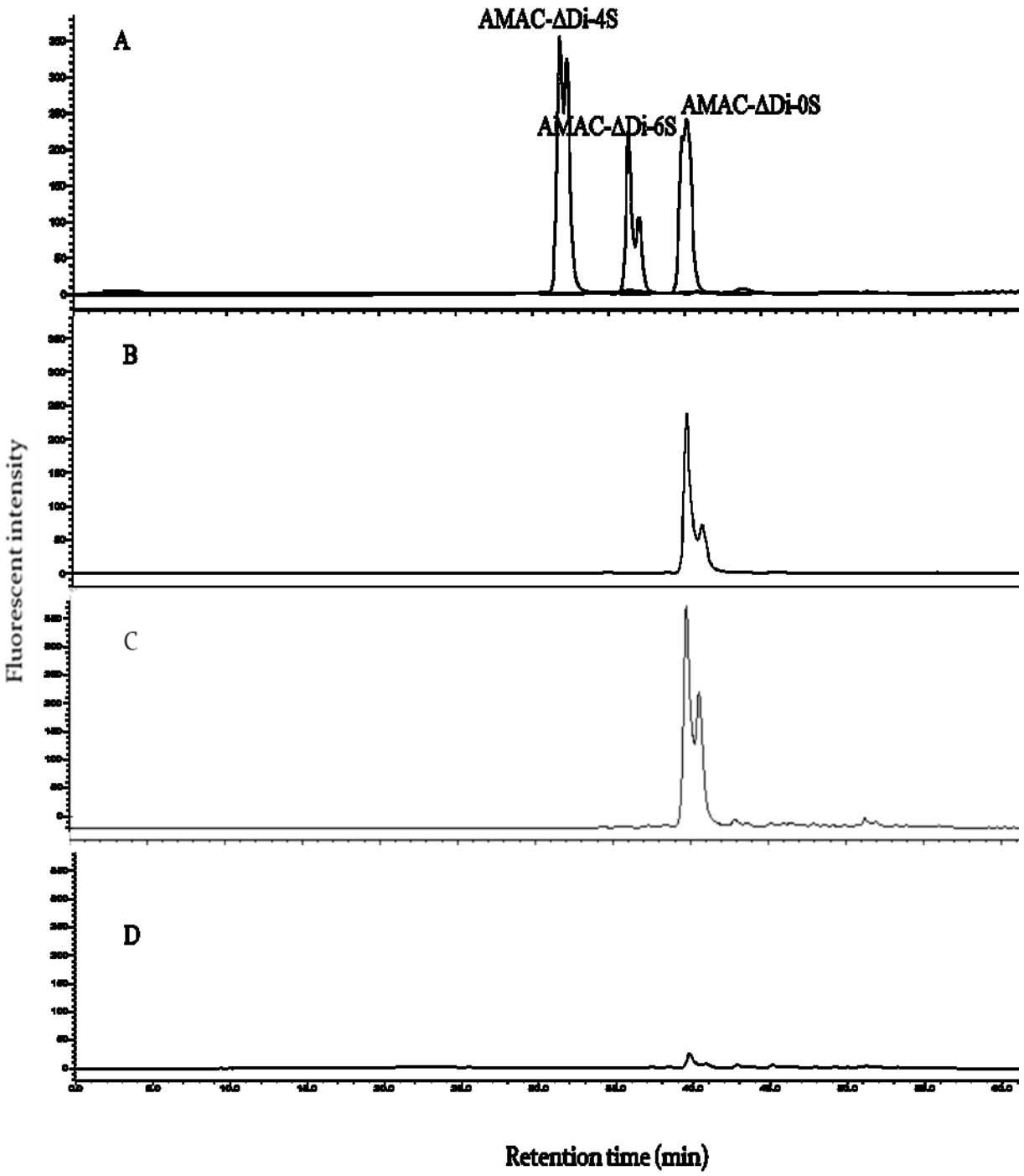

Figure 5. 5. Chromatogram of unsaturated disaccharides fluorotagged with AMAC: A) Δdi-4S, Δdi-6S, and Δdi-0S standard; B) Combined (acid-36h and alkaline-60h) treated CS; C) Acid (60h) treated CS; C) Alkaline (60h) treated CS.

5.3.4. Antioxidant capacity of the hydrolyzed CS

The antioxidant capacity of polysaccharides depends on their structural characteristics, such as chemical composition (i.e. sulfation and acetylation), molecular weight, types of glycosidic linkage and conformation. Among these characteristics, the molecular weight was one of the most important structural features of a polysaccharide. A number of reports suggest that polysaccharides with lower molecular weights have more reductive hydroxyl group terminals (per unit mass basis) to accept and eliminate the free radicals (Chen, Tsai, Huang and Chen, 2009; Yan, Li, Wang, Leung, Wang and Wu, 2009; Wang, Hu, Nie, Yu and Xie. 2015). In the previous study reported in chapter 3, the CS-oligosaccharide (< 10 kDa) derived from enzymatically-hydrolyzed CS had a superior reducing power as well as better free radical scavenging activities (chapter 3). It is therefore of interest to understand if the chemically depolymerised CS results in the same effect. Based on previous results, the treatments that were chosen for antioxidant assays were: Acid hydrolysis-60 h, Alkaline hydrolysis-60 h and the combined treatment acid-36 h + alkaline-60 h.

5.3.4.1. DPPH radical assay

The scavenging activities of the chemically depolymerised CS on DPPH radicals are shown in Figure 5.6A, at a testing concentration at 1.0 mg/mL. All three treatments showed significantly greater scavenging activities as compared to the CS control. This suggests that both the acid and alkaline treatments through either hydrolytic or β-elimination cleavages improved CS scavenging activities. The greatest DPPH ($p < 0.05$) level was observed in the combined treatment followed by the acid-hydrolyzed CS (74 ± 3% and 78 ± 5%, respectively), which was not significantly different as compared to the positive control ascorbic acid (Figure 5.6A). The alkaline treated CS had the least ($p < 0.05$) DPPH scavenging activity among the three treated samples. The depolymerisation through acid hydrolysis increased DPPH scavenging likely due to the increased

generation of reducing hydroxyl groups at the terminal end of CS chains. Both the acid and the combined treatments showed a greater degree of depolymerisation and desulfation (Figure 5.4). Although the negatively charged sulphate groups are speculated to trap free radicals in an electrostatic manner (Balt et al., 1983), the depolymerisation effect seems to be more relevant in this study. These results concur with the work by Yan et al. (2009), who indicated that a high degree of sulfation in alkaline treated CS was not relevant for improving the DPPH scavenging activities. Another possible reason on why the combined treatment increased the radical scavenging activity may be due to not only an increasing amount of –OH groups as a consequence of the acid hydrolysis, but also free $–NH_2$ groups produced due to the deacetylation under alkaline conditions. Therefore, oligosaccharides with an increased number of –OH and NH_2 groups could be produced during this combination. This has the potential to increase both the reduction and radical scavenging ability of the CS hydrolysate. An aldose-amine structure (amino group at the C2 position next to the carbonyl group; i.e. galactosamine) is rather unstable and can go through a series of modifications (i.e. non-enzymatic browning) to produce compounds that have both radical and reducing potential (Hrynets, Ndagijimana and Betti, 2015; Hong and Betti, 2016). The CS hydrolysates produced under alkaline conditions showed a darker-brown color to the naked eye (data not shown).

5.3.4.2. Ferric ($Fe3^+$) reducing activity and iron chelating capacity

Ferric reducing and iron chelating activities were also used to evaluate the antioxidant potential. It is important to know how the chemically-depolymerised CS reacts with ferric (Fe^{3+}) and ferrous (Fe^{2+}) ions for the ferritin study in the Caco-2 cell models. Fe^{3+}, as the majority of dietary non-heme iron, is essentially insoluble and is not bioavailable, while the Fe^{2+} is readily absorbed but easily oxidized into the insoluble Fe^{3+} form. Thus, both the solubility and the

oxidation state of iron under the influence of the tested compounds determines the extent of iron absorption and ferritin formation in the Caco-2 cells.

The ability of reducing Fe^{3+} to Fe^{2+} in the treated CS samples was analyzed and is presented in Figure 5.6B. The native CS and ascorbic acid were used as negative and positive controls, respectively. Among the three chemical treatments, only the combined one (acid and alkaline) showed a significantly greater amount of Fe^{2+} (34 ± 3 mmol) reduced from the total of 50 mmol Fe^{3+} as compared to the negative control ($p < 0.05$). As mentioned in the previous section, acid hydrolysis resulted in more CS depolymerisation and desulfation with reduced deacetylation, while alkaline hydrolysis effectively removed the *N*-acetyl groups but resulted in less depolymerisation and desulfation on the CS polysaccharide chains. Neither the acid nor alkaline treatments produced oligosaccharides with ferric reducing activities like the positive control ascorbic acid. On the other hand, the combined acid-alkaline treatment resulted in a greater capacity ($p < 0.05$) to reduce Fe^{3+} to Fe^{2+} as compared to the acid or alkaline treatment alone. As explained in the previous section, the concomitant production of CS oligosaccharides along with an increased generation of free $–NH_2$ groups in the combined treatment can generate unstable oligosaccharides capable of non-enzymatic browning reactions resulting in the production of "reductones", compounds with increased reduction potential toward metal ions (Hrynets et al., 2015). Although the alkaline treated CS also showed a large degree of deacetylation (Figure 5.4B), no release of disaccharide unit was detected and there were limited depolymerisation on the CS polysaccharides chain as evidenced by the results from cellulose acetate electrophoresis and RP-UPLC (Figure 5.3 and 5.5).

The ability to bind transition metals is another way to estimate the tested compound's antioxidant potential by stabilizing this reactive compound and making it less prone to initiating

radical mediated oxidation, known as the Fenton reaction (Wang et al., 2015). The CS is proposed to form a stable complex with positively charged transition metal ions, such as Cu^{2+} and $Fe^{2+,}$ since it has negatively charged functional groups that can participate in iron linkage. In addition, iron complexation with CS has also been considered as a strategy for increasing iron solubility and uptake by the Caco-2 cells (Miquel and Farré, 2007). In this study, the alkaline-treated CS showed the best chelating ability against Fe^{2+} ions among the three treatments, indicating that the chelating effect might be impacted by the increased number of sulfate groups remaining on the CS chain under an alkaline environment. In addition, the alkali-aided deacetylation exposes the primary amino groups that may also contribute to the chelating capacity. Wang et al. (2015) reported that the structure of compounds containing more than one of following functional groups, that is -OH, $-SO_3$, -COOH, -C=O and NH_2, favors chelating ability. This also explains the relative large chelating capacity of the combined treated CS as compared to the acid-treated one (Figure 5.6C). Although the combined treated CS lost more than half of the sulfate groups (Figure 5.4C) after the acid pre-treatment, the following alkaline deacetylation may have resulted in more amino and carbonyl groups associated with superior chelating capacity. In summary, the combined (acid and alkaline) treatment produced CS oligosaccharides that showed superior reducing capacity but moderate iron chelating activity. The possible mechanism may involve conformational changes in structure of CS polysaccharides by acid-assisted depolymerisation to cause an emerging increase in the amount of -OH, -C=O and NH_2 functional groups by alkaline-aided deacetylation, thus influencing their antioxidant capacity.

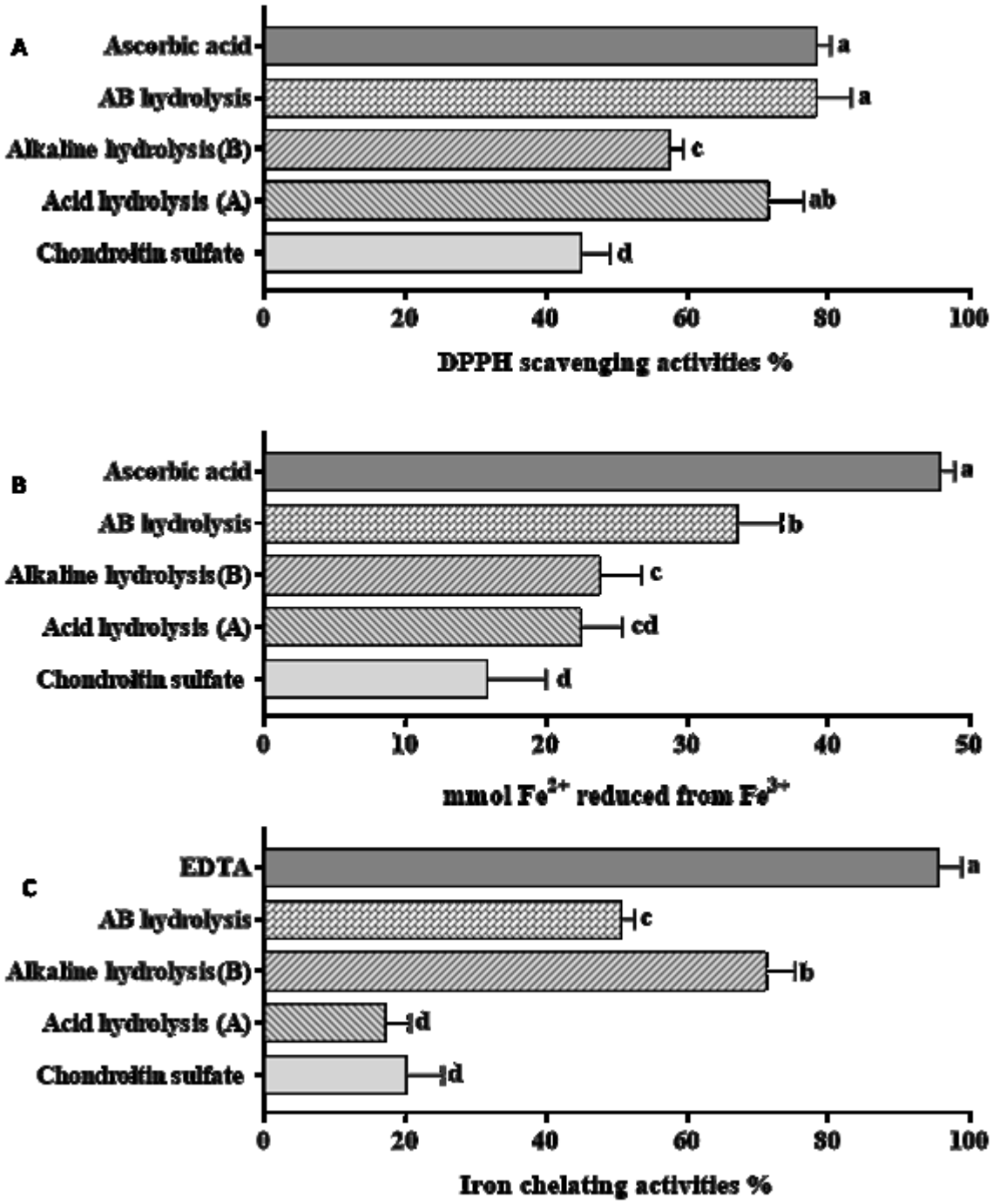

Figure 5. 6. DPPH scavenging activities (A), iron reducing activities (B) and iron chelating activities (C) for the acid (60 h), alkaline (60 h) and combined (acid: 36 h and alkaline: 60 h) treated CS. Values are expressed as mean ± standard deviation (n=3); $p<0.05$. Sample concentration of 1.0 mg/ml; 50 mM of Fe^{3+} was used.

5.3.5. Effect of the chemical hydrolyzed CS on ferritin formation in Caco-2 cells

The *in vitro* digestion/Caco-2 cell culture model is useful for evaluating iron absorption at the human intestinal level (Quintaes, Barberá and Cilla, 2015). Figure 5.7 shows the ferritin synthesis by Caco-2 cells, an indirect measurement of iron bioavailability (ng ferritin/mg cell protein), for chemically-treated CS samples with the presence of $FeCl_3$ as their iron source. Ascorbic acid and untreated CS were used as positive and negative controls, respectively. Both the combined and acid treated CS samples induced greater ferritin formation than the alkaline treated CS. No significant difference was found between the alkaline treated CS and the untreated CS negative control. Based on the antioxidant studies in the previous section, the chemically treated CS and acid treated CS possessed more DPPH radical scavenging activity, but less iron chelating activity as compared to the alkaline treated CS. This indicates that the enhancing effect on Fe uptake is mainly affected by the level of depolymerisation and the reducing capacity of the chemically-treated CS, rather than the degree of sulfation and the metal chelation ability. This agrees with the previous study of chapter 3 where the improvement of iron uptake by Caco-2 cells was positively related to the radical scavenging activity and reducing power (chapter 3). However, the study found that enzymatically hydrolyzed CS induced the production of ferritin at a level of 200 ng/ mg cell protein, which is greater than the amount found in this study (150 ng/mg cell protein). Hence, enzymatically-produced CS oligosaccharides is a better option to obtain CS-oligosaccharides with greater ability to increase iron uptake and thus potentially increase the iron bioavailability. Furthermore, the loss of sulfate groups might negatively affect the bioactivity of CS oligosaccharides in relation to their ability to fight osteoarthritis.

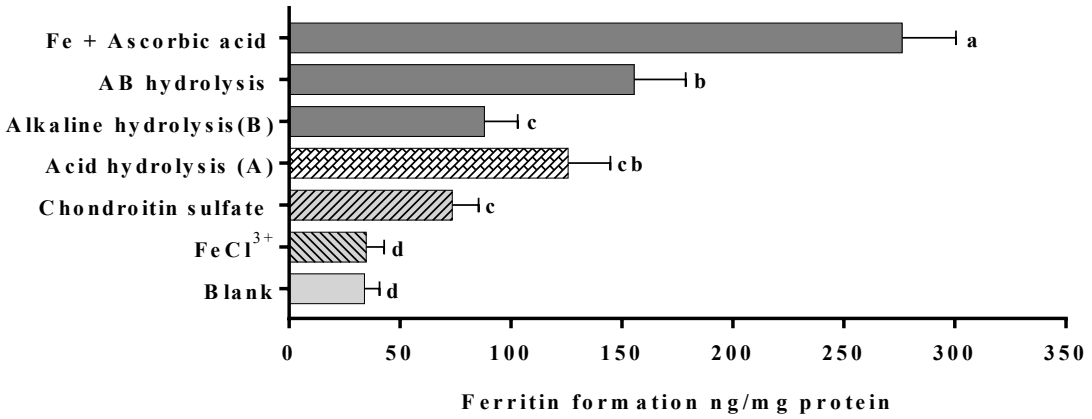

Figure 5. 7. Ferritin levels in Caco-2 cells exposed to the acid (60 h), alkaline (60 h) and combined (acid: 36 h and alkaline: 60 h) hydrolyzed CS. Samples (final concentration= 1.0 mg/mL) were mixed with $FeCl_3$ (final concentration=41.7 µmol/L) and subjected to *in vitro* digestion. Values are expressed as mean ± standard deviation (n=3); $p<0.05$. AA: ascorbic acid.

5.4. Conclusion

This study provides evidence that CS can depolymerise through a number of pathways resulting in decomposition products (i.e. oligosaccharides) with different structural characteristics. Under alkaline conditions, β-elimination at the glycosidic linkage was found to be limited. Little desulfation was observed but a large degree of deacetylation is taking place in the alkaline-treated CS sample. Under acidic conditions, the molecular weight decreased through hydrolysis of glycosidic bond resulting in an increased number of hydroxyl groups, but a substantial loss of the sulfate groups and insufficient deacetylation was observed. Superior antioxidant properties were observed in acid and combined treated samples. The chemical modification on the CS structure conformations also enhanced its effect on the Fe uptake by Caco-2 cells, especially for acid and combined treated CS samples. The use of acid hydrolysis seems to be a simple method to obtained CS-oligosaccharides with antioxidant and enhancing effect on Fe uptake. However, these structure alterations such as loss of sulfate groups may negatively affect other existing therapeutic use of CS. Future studies are required to identify other therapeutic features such as anti-arthritis and anti-tumor bioactivities for these chemically- hydrolyzed CS products.

Chapter 6. Conclusions, implications, and future directions

This doctoral research is the foundation for using animal-derived sulfated GAGs to develop a multifunctional food supplement to treat anemia. Firstly, this work represents a novel food-grade process to successfully produce high-quality animal sulfated GAGs derived from meat industry processing by-products. Most of these meat by-products contain a large amount of connective tissue, rich in both GAGs and collagen. The current extraction methods only extract collagen and the GAGs are usually discarded. Not only are these being underutilized, but they also generate unnecessary waste. The ultrafiltration technology applied in this food-grade process offers an economical way for the co-extraction of GAGs and collagen peptides (Figure 3.1), translating to an increased extraction margin and better utilization of the meat by-products. Secondly, this work contributes to the understanding of the structure-activity relationship of these sulfated polysaccharides made by enzymatic depolymerisation. These depolymerized sulfated GAG oligosaccharides have a smaller molecular weight enabling them to complex with iron more readily and thus be better absorbed by enterocytes. In addition, they possess multiple functional groups, which have better antioxidant activities and enhancing effects on iron uptake by Caco-2 cells compared to their polysaccharide forms. This not only confirms the Fe absorption enhancing effect possessed by animal sulfated GAGs, but also suggests a possible means to increase this effect for them. A greater knowledge of these effects and their mechanisms is important for designing future diets with an enhanced iron bioavailability, as well as for developing food products that appeal to health-conscious consumers. Therefore, the potential use of these sulfated GAG-oligosaccharides to fortify food with iron was evaluated (Chapter 4). Skim bovine milk, in this case, was chosen as a naturally designed food matrix that represents a food and/or diet with poor iron bioavailability suitable for iron fortification. The promoting effect of sulfate GAG-oligosaccharide on Fe uptake was pronounced in this food matrix. Again, this provides evidence that these sulfated GAG-

oligosaccharides can serve as a food fortification/supplement system that improves dietary non-heme iron bioavailability. In addition, it is the first time that the effect of milk oligosaccharides was evaluated on iron uptake specifically, an effect which was similar to that of whey. More importantly, the identification of milk GAGs, specifically (CS/DS structures) in this low molecular weight oligosaccharide fraction, suggests it may contribute to the prebiotic effect of milk oligosaccharides, which should be further studied. Finally, it contributes to finding a more economical way to produce sulfated GAG oligosaccharides by chemical depolymerisation.

This doctoral work shows future promise for the application of sulfated GAG oligosaccharides as food supplements that are both antioxidants and promote iron bioavailability. However, improvements still could be made to provide a more complete understanding of the subject. For example, the transport passage of sulfated GAG oligosaccharides through enterocytes was not investigated. Whether the GAG-iron complex could be internalized via endocytosis still needs to be confirmed. Although the cell cytotoxicity (MTT) test was satisfied at the applied concentration of sulfated GAG and their derivatives, the cell oxidant stress level was not assessed after the addition of extrinsic iron. Here remains a concern that an increase in soluble iron content with GAG-iron fortification could increase free radical production in the colon to a level that causes mucosal cell damage. Furthermore, other problems associated with food fortification with iron, in general, were not addressed, such as sensory changes and sediment formation, need to be investigated. Also, a more suitable non-enzymatic depolymerisation method with less effect on the GAG's functional groups (i.e. sulfate groups) should be evaluated.

For research emerging from this thesis, the collaboration with Dr. Bourque's group from Depts. Anesthesiology and Pain Medicine and Pharmacology Women and Children's Health Research Institute (University of Alberta) using an in vivo model for anemic rats has been currently

under investigation. The concerns of elevated oxidative stress level after oral administration of sulfated GAG-oligosaccharides with iron may be addressed in this in vivo study. The effect of sulfated GAG-oligosaccharides on Fe uptake in a different food matrix, such as iron-fortified bread and pasta, may also be evaluated. The new and emerging technology using subcritical water may provide a more suitable non-enzymatic depolymerisation method for the mass production of bioactive sulfated GAG oligosaccharides. Also, other cationic minerals with critical biological functions in the body, such as zinc and calcium, could also be studied regarding their GAG-mediated enhancement of absorption.

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Appendix A

Weight of the extracted glycosaminoglycans in different fractions (low and high sulfate) and their recovery (W/W) from 50 g defatted dry chicken skin, meat and cartilage (mean ± standard deviation, n=3).

Material	Low sulfate (g)	High sulfate (g)	Total (g)	% in the dry raw material	Recovery %
Chicken Skin	0.46±0.04	0.35±0.02	0.81±0.02	1.6±0.02	71.2±0.05
Chicken Meat	0.22±0.06	0.31±0.02	0.53±0.03	1.1±0.06	59.2±0.06
Chicken Cartilage	0.44±0.02	3.2±0.03	3.65±0.03	7.3±0.09	74.7±0.05

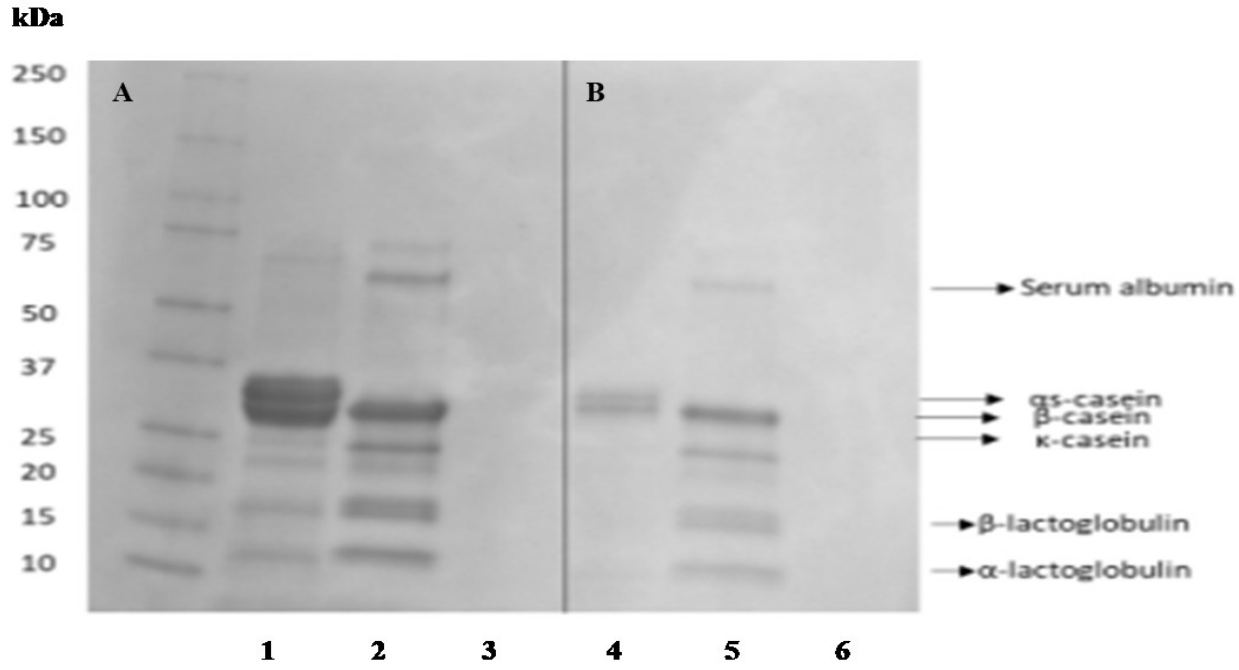
Appendix B

Analysis of the extracted glycosaminoglycan from broiler chicken skin, meat and cartilage in low and high sulfated fractions (mean ± standard deviation, n=3).

	Low sulfated fraction		High sulfated fraction	
	Skin	Cartilage	Skin	Cartilage
Peptides (µg/mg)	78.2±6.9	55.9±4.3	8.6±2.6	7.1±0.9
Sulfate/uronic acid (weight ratio)	0.21	0.39	0.73	0.96
peptides/uronic acid (weight ratio)	0.97	0.76	0.04	0.03

Appendix C

Protein profile of each skim bovine milk fractions before (A) and after (B) *in vitro* digestion. A1, A2, A3 are casein, whey and lactose before digestion; B4, B5, B6 are casein, whey and lactose after *in vitro* digestion.



Appendix D

Fluorophore derivatization of unsaturated non-sulfated disaccharide (as an example) of CS with AMAC in the presence of reductive amination by cyanoborohydride.