CHAPTER 2

Nutraceutical Functionalities of Polysaccharides from Marine Invertebrates

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Contents

Abstract

Many researchers are seeking functional materials from marine resources. These marine resources can be used as traditional food additives, and specifically, these are based on polysaccharides. To date, there is a big opportunity to develop new high-value added products with indispensable functional characteristics, which can be used in nutraceuticals either as additives or supplements. Also, a crossover in the pharmaceutical market may be established. Some glycosaminoglycans (GAGs) mimetic-type molecules are already being utilized in the field of nutrition as well as in the cosmetics industry. This chemical is used as a dietary supplement to maintain the structure and function of cartilages, for the relief of pain caused by osteoarthritic joints, and can also be used as an anti-inflammatory agent. Recently, in relation to the prevalence of mad cow disease and avian influenza, the production of GAGs from marine invertebrates offers new market opportunities as compared with that obtained from bovine or avian livestock.

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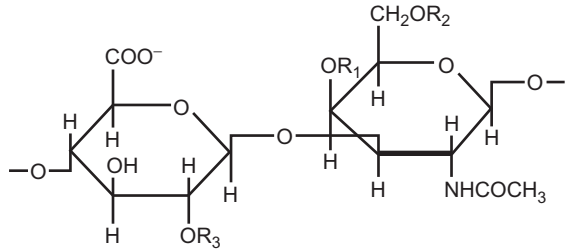
I. INTRODUCTION

The discovery of *O*-linked β-*N*-acetylglucosamine (*O*-GlcNAc) more than 28 years ago disproved the long-held dogma that protein glycosylation is restricted to the luminal compartments of the secretory machinery and to the cell surface and extracellular matrix (Hart *et al*., 1989). Early studies of *O*-GlcNAc's subcellular localization in rat hepatocytes established that it is highly concentrated at the nuclear envelope, particularly at the nuclear pore complex, but is also abundant and widespread within chromatin (Holt and Hart, 1986). Aside from the biosynthetic intermediates, *O*-GlcNAc modification, where β-linked GlcNAc is attached to Ser or Thr residues of cytosolic/nuclear proteins, is the best-known example of cytosolic/nuclear glycosylation. This modification is found to occur exclusively in the cytosol or nucleus, and unlike other types of glycosylation, it remains as a single GlcNAc residue through a β-glycosidic linkage to Ser/Thr residues and is not elongated further by the addition of other sugars (Altmann *et al*., 2001). The biological functions of *O*-GlcNAcylation in cytosolic events can be summarized as follows: (1) it inhibits protein phosphorylation on Ser/Thr residues, by reciprocal site occupation (Wang *et al*., 2007); (2) it affects protein degradation, by influencing PEST sequences known as the rapid degradation signal motif (Cheng *et al*., 2000) or by directly modifying the proteasome complex to decrease the proteasomal function (Sumegi *et al*., 2003); (3) it regulates intracellular localization of the carrier proteins (Guinez *et al*., 2005); (4) it is involved in protein interaction, some *O*-GlcNAcylated transcription factors are known to interact for transactivation (Hiromura *et al*., 2003); and (5) it affects the activity of transcription factors or repressors (Yang *et al*., 2002).

Glycosaminoglycans (GAGs) are large, complex carbohydrate molecules that are linear, negatively charged, and composed of disaccharide repeating units (Gandhi and Mancera, 2008). These molecules are sometimes known as sulfated mucopolysaccharides because of their viscous, lubricant properties, as found in mucous secretions. GAGs are present on all animal cell surfaces and in the extracellular matrix (Medeiros *et al*., 2000). Owing to the variability in sulfate substitution, all GAGs display considerable sequence heterogeneity, and it is believed that structural differences are responsible for highly specific interactions of GAGs with other macromolecules (Coombe and Kett, 2005). Their strategic location and highly charged nature make them important biological players in cell–cell and cell–matrix interactions that take place during normal and pathological events, including organogenesis in embryonic development, cell recognition, adhesion, migration, regulation of growth factor action, wound repair, lipid metabolism, neural development and regeneration, and initiation and modulation of inflammation (Laabs *et al*., 2005; Sugahara *et al*., 2003;

Taylor and Gallo, 2006; Whitelock and Iozzo, 2005). GAGs include chondroitin sulfate (CS), dermatan sulfate (DS), keratan sulfate (KS), heparin, and heparan sulfate (HS; Nadanaka and Kitagawa, 2008; Nader *et al.*, 2004; Volpi, 2007; Yamada and Sugahara, 2008). Their disaccharide repeating units are composed of hexosamine (D-glucosamine or D-galactosamine) and either hexuronic acid (D-glucuronic or L-iduronic acid) or galactose (as in KS). DS, HS, and heparin contain both glucuronic acid and iduronic acid units, whereas CS has glucuronic acid as the only hexuronic acid. In the tissue, GAGs are covalently bound to a protein core forming a structure known as proteoglycan (Didraga *et al.*, 2006).

CS, in particular, is composed of alternate sequences of D-glucuronic acid and differently sulfated residues of *N*-acetyl-D-galactosamine linked by β(1→3) bonds. Depending on the disaccharide nature, CS with different carbohydrate backbones is known. Chondroitin-4-sulfate, CSA, is constituted by 4-sulfated disaccharides [(1→4)-*O*-(D-glucopyranosyluronic acid)-(1→3)-*O*-(2-*N*-acetamido-2-deoxy-D-galactopyranosyl-4-sulfate)]. Chondroitin-6-sulfate, CSC, is mainly composed of a disaccharide unit sulfated in the position 6 of the *N*-acetyl-D-galactosamine [(1→4)-*O*-(D-glucopyranosyluronic acid)-(1→3)-*O*-(2-*N*-acetamido-2-deoxy-D-galactopyranosyl-6-sulfate)] (Fig. 2.1). However, even if the known CS samples are mainly composed of various percentages of these two kinds of disaccharide units, monosulfated in position 4 and monosulfated in position 6 of the *N*-acetyl-D-galactosamine, disaccharides with a different number and position of sulfate groups can be located, in various percentages, within the polysaccharide chains. For example, the nonsulfated disaccharide is



$R_1 = R_2 = R_3 = H$: nonsulfated chondroitin
$R_1 = SO_3^-$ and $R_2 = R_3 = H$: chondroitin-4-sulfate, CSA
$R_2 = SO_3^-$ and $R_1 = R_3 = H$: chondroitin-6-sulfate, CSC
$R_2 = R_3 = SO_3^-$ and $R_1 = H$: chondroitin-2,6-disulfate, CSD
$R_1 = R_2 = SO_3^-$ and $R_3 = H$: chondroitin-4,6-disulfate, CSE
$R_1 = R_3 = SO_3^-$ and $R_2 = H$: chondroitin-2,4-disulfate, CSB
$R_1 = R_2 = R_3 = SO_3^-$: trisulfated chondroitin

FIGURE 2.1 Structures of disaccharides forming chondroitin sulfate. Adapted from Volpi (2007). With permission from Wiley Publisher.

generally present in low amounts in the CS backbone, while the monosulfated disaccharide in position 2 of the glucuronic acid is very uncommon in this natural polymer. On the contrary, disulfated disaccharides having two sulfate groups *O*-linked in various positions, such as 2 of D-glucuronic acid and 6 of *N*-acetyl-D-galactosamine (disaccharide D), or in position 4 and 6 of *N*-acetyl-D-galactosamine (disaccharide E) or 2 of the uronic acid and 4 of *N*-acetyl-D-galactosamine (disaccharide B; Fig. 2.1) may be present in the CS backbone in various percentages in relation to specific animal sources. Further, these heterogeneous structures are responsible for the various and more specialized functions of these polysaccharides (Volpi, 2007).

The CS, such as the other GAGs, is linked to the core protein through a tetrasaccharide junction, composed of xylose, galactose, and glucuronic acid. The whole assemblage, is generally referred to as proteoglycan and is widely distributed in connective tissues both in the cellular membrane (Yanagishita and Hascall, 1992) and in the extracellular matrix (Iozzo, 1998). In this last case, aggrecan is the principal CS proteoglycan in the cartilage, where it is found associated with hyaluronic acid, collagen, and other proteins; this multimeric aggregate confers to the tissue its hydrate gel-like and elastic characteristics. The role of aggrecan is to draw water into the extracellular matrix which swells and expands acquiring its compressive resilience. Aggrecan molecular modifications are regulated by many cellular and extracellular events; however, individual age appears to play an important role on cartilage composition, influencing the sulfation pattern of chondroitin and proteolytic cleavage of aggrecan yielding an extracellular matrix with a reduced amount of poorly sulfated chondroitin. These events play a central role in joint degenerative diseases such as osteoarthritis and rheumatoid arthritis (Hardingham and Bayliss, 1990). In addition, cartilage breakdown products with antigenic properties are observed and their release into synovial fluid induces synovial inflammation (Volpi, 2006). Pharmacological management of the osteoarthritis is based on the use of analgesics, steroidal and nonsteroidal anti-inflammatory drugs, in combination with CS polysaccharides, resulting in the increasing demand of this material and in the search of new sources which are able to provide this polysaccharide with a sulfation pattern compatible with human physiology (Gargiulo *et al*., 2009).

II. SOURCES OF POLYSACCHARIDES

A. Sea cucumbers

The body wall of sea cucumber is easily and greatly hardened by heat, acid treatment, or even by handling stimulation under physiological conditions. Chemical and rheological analyses of the body wall have not

provided any clues about the mechanism involved (Motokawa, 1981, 1982). It has been argued that the GAG of the sea cucumber's (*Stichopus japonicus*) body wall is involved in the cation-dependent change of the toughness of its connective tissue (Kariya *et al*., 1990). Also, it has been shown that the specific viscosity of the GAG solution was affected differently depending on the valence and species of cation. Interestingly, the mode of change in the viscosity of this GAG solution was quite different from those reported for known GAGs, such as CS and DS. The sea cucumber GAG is not digested by both chondroitinase ABC (EC 4.2.2.4) and AC (EC 4.2.2.5), although in addition to neutral sugar (fucose), the GAG has a sugar backbone identical to CS which contains glucuronic acid (GlcA) and *N*-acetylgalactosamine (GalNAc).

Such unique physicochemical properties of the sea cucumber GAG seem to be related to its unusual structure. Vieira and Mourao (1988) demonstrated the presence of two fucopyranosyl residues linked glycosidically through position (1→4) and/or (1→2) for the fucose branch in the GAG from the sea cucumber *Ludwigothurea grisea*. The two fucopyranosyl residues of the fucose branch were linked (1→3) only. In addition, about 20% of total fucose branches were linked glycosidically through the *O*-3 position of a GlcA moiety (the remaining 80–90% were assumed to be linked through GalNAc). Fucose-branched chondroitin sulfate E (CSE) was prepared from the body wall of sea cucumber, *S. japonicus* (Kariya *et al*., 1997). The purified GAG was chemically desulfated, followed by carboxyl reduction. Intact, desulfated, and desulfated/carboxyl-reduced GAGs fractions were subjected to per-*O*-methylation. GC-MS analyses of the resultant partially methylated alditol acetates demonstrated that fucose branch is formed by two fucopyranosyl residues linked glycosidically through position (1→3), and that the fucose glucuronic acid branches are almost equimolar.

The sea cucumber, *Apostichopus japonicus* is a traditional food and of high demand in China. In recent years, farming and sea ranching of *A. japonicus* have grown into a prosperous economic sector in northern China, where 2–3 billion juveniles and 60,000 ton of sea cucumbers were produced each year (Yang *et al*., 2005). The rapid expansion of sea cucumber farming and high farming intensity resulted in serious diseases; mainly skin ulceration syndrome, which is highly infectious and lethal to this animal (Zhang *et al*., 2006). Application of antibiotics and chemicals in aquaculture leads to the spread of drug-resistant pathogens and deleterious environmental consequences (Reilly and Kaferstein, 1997; Sun *et al*., 2007). Therefore, finding alternatives to antibiotics and chemicals is very urgent and important for *A. japonicus* farming. Using immunostimulants is a promising area in aquaculture because they are biocompatible, biodegradable, environment-friendly, and safe for humans (Luo, 2007). Among potential immunostimulants, β-glucan and mannan

oligosaccharide (MOS) were used with many kinds of fish diets (Chansue *et al.*, 2000), and both have commercial products available for aquaculture, such as MacroGard (Biotec Pharmacon, Tromso, Norway), DVAQUA (Diamond V Mills, Inc., IA, USA), and Bio-Mos (Alltech, USA). Many feeding trials and *in vitro* tests have shown that β-glucan and MOS are able to enhance the resistance of aquatic animals against infections as well as immune capacities such as phagocytosis, superoxide anion production, and lysozyme activity in shrimps and fishes (Dugenci *et al.*, 2003; Zhao *et al.*, 1990).

Collagen fibrils from the dermis of the sea cucumber *Cucumaria frondosa* are aggregated *in vitro* by the dermal glycoprotein stiparin (Trotter *et al.*, 1996). Under physiological ionic conditions, stiparin appears to be both necessary and sufficient to cause fibrils to aggregate (Trotter *et al.*, 1997). Trotter *et al.* (1999) reported the initial biochemical and biophysical characterization of a sulfated glycoprotein from *C. frondosa* dermis that binds stiparin and inhibits its fibril-aggregating activity. This inhibitory glycoprotein, which has been named ''stiparin-inhibitor,'' has the highest negative charge density of all the macromolecules extracted from the dermis. SDS-PAGE reveals three ~31 kDa bands which stain with Alcian blue but not with Coomassie blue. Analytical ultracentrifugation indicates a native molecular weight of 62 kDa. Transmission electron microscopy of rotary-shadowed molecules shows curved rods about 22 nm long. The glycoprotein does not bind collagen fibrils but does bind with stiparin in a 1:1 stoichiometry. The binding of stiparin-inhibitor to stiparin prevents the binding of stiparin to collagen fibrils. The carbohydrate moiety produced by papain digestion of the glycoprotein retains all of its inhibitory activity. The carbohydrate moiety of the inhibitor is dominated by galactose and sulfate. The metabolism of both orally and parenterally administered exogenous GAGs is not well understood. An understanding of this metabolism is essential in exploiting new therapeutic applications (Dawes *et al.*, 1991). The GAG isolated from a holothurian (*S. japonicus*) is a unique fucose-branched CS that has an anticoagulant activity (Table 2.1; Vieira and Mourao, 1999). An undesirable characteristic of this GAG is that it induces platelet aggregation (Li and Lian, 1988). In an effort to reduce this property, a partially depolymerized holothurian glycosaminoglycan (DHG) has been prepared by oxidative depolymerization with hydrogen peroxide (Suzuki *et al.*, 1991). DHG, a fucosyl CS chain, was isolated from sea cucumber and was intravenously and orally administered to experimental animals. After intravenous injection, clearance of DHG, as measured by postcolumn HPLC, displayed complex kinetics that were not dose dependent. DHG was excreted unchanged in the urine. No degradation products of DHG were detected by either gel filtration or anion exchange HPLC at any time in the plasma, indicating

TABLE 2.1 Methylation analysis of intact, desulfated, and desulfated/carboxyl-reduced fractions prepared from the sea cucumber *S. japonicas* GAG

			Molar ratio[c]		
Peak	PMAA[a]	t_R[b]	Intact	Desulfated	Desulfated/ carboxyl-reduced
1	2,3,4-Fuc	1.00	ND[d]	0.21 (21)	0.21 (21)
2	2,4-Fuc	1.19	0.16 (16)[e]	0.45 (45)	0.25 (25)
3	2,3-Fuc	1.22	0.08 (8)	0.03 (3)	0.04 (4)
4	3,4-Fuc	1.33	0.23 (23)	0.07 (7)	0.03 (3)
5	3-Fuc	1.41	0.17 (17)	0.05 (5)	0.03 (3)
6	Fuc	1.43	0.18 (17)	0.04 (4)	0.04 (4)
7	2,3,6-Glc	1.50	ND	ND	0.15 (15)
8	2,6-Glc	1.64	ND	ND	0.04 (4)

Adapted from Kariya *et al.* (1997). With permission from Elsevier Publisher.
[a] Partially methylated alditol acetate with methoxy groups at the positions shown.
[b] Retention time on a SP2330 capillary column relative to 2,3,4-tri-*O*-methyl-fucitol.
[c] The molar ratios were based on the peak area.
[d] Not detected.
[e] Numbers in parentheses represent percentage distribution of PMAAs.

that administered DHG cannot undergo catabolic degradation in mammals. Anion exchange chromatographic behavior of DHG excreted into the urine after oral administration showed that partial desulfation might occur through intestinal absorption. After oral administration of DHG (50 mg/kg), 0.1% of the dose was found in the urine collected for 24h. More than 5% of intravenously administered DHG (1mg/kg) was excreted into the urine in 24h. These results suggest that orally administered macromolecules such as DHG are absorbed in the gastrointestinal tract (Imanari *et al.*, 1999).

CSE obtained from sea cucumber markedly enhanced plasminogen activation by tissue plasminogen activators (t-PAs) and urinary plasminogen activator (u-PA) *in vitro*; in the presence of 10mg/ml of CSE, the potentiation factors of single chain of t-PA, two chains of t-PA, and u-PA were 400, 140, and 130, respectively. Though the potentiation activity of CSE gradually decreased when it was depolymerized by chondroitinase ABC, the specific disaccharide from CSE still showed significant activity. GAG from sea cucumber, which possesses a very similar core structure to CSE, but has additional sulfated fucose branches, exhibit very weak activity. The minimal structural requirement in CSE to enhance plasminogen activation by plasminogen activators is GlcUAβ1-3GalNAc(4S,6S) and that additional branching sugars deter the activity (Sakai *et al.*, 2000).

B. Ascidians

Ascidians are marine invertebrates and the closest living relatives of vertebrates. Tadpole-type larvae of ascidians represent the simplest chordate body plan (Satoh and Jeffery, 1995). However, ascidians do not produce cartilage or mineralized bone at any stage of their lives. These types of endoskeleton have developed in vertebrates after their divergence from ascidian groups. Therefore, ascidians are expected to have only a minimal number of genes involved in the enzymatic modification of GAGs. Proteoglycans are glycoproteins consisting of many unbranched polysaccharide chains associated with a core protein. CS-containing proteoglycans (CSPGs) are ubiquitously found in the extracellular matrix and on the cell surface (Tetsukawa *et al*., 2010). In vertebrates, CSPGs are major components of cartilage where they mainly serve as a cushion. The cell adhesion molecule integrin requires CSPG on the cell surface to bind fibronectin (Moyano *et al*., 1999). In some cases, CSPGs promote neurite outgrowth (Clement *et al*., 1998), while in others, they function as a repulsive signal to inhibit axonal growth (Masuda *et al*., 2004). A type of CS binds to a heparin-binding neurotrophic factor, midkine, and inhibits its function (Ueoka *et al*., 2000).

The antithrombotic effect of *Styela plicata* heparin was evaluated in two models of thrombosis. One of the models, widely used for venous thrombosis, is based on the evaluation of thrombus formation induced by two major factors, activation of coagulation and stasis (Vogel *et al*., 1989). The resultant venous thrombus is composed of fibrin and red blood cells. In the arteriovenous shunt model, thrombosis is initiated by platelet adherence to a silk thread anchored in the shunt, and both activation of platelets and coagulation contribute to thrombus formation. *S. plicata* heparin was much less potent to inhibit thrombosis than mammalian heparin, and complete thrombus inhibition was not achieved, even at a concentration of fourfold higher than that required for mammalian heparin to inhibit 100% thrombosis. Therefore, heparin preparations obtained from the body of *S. plicata* would have a safer therapeutic action in the treatment of arterial thrombosis than mammalian heparin (Santos *et al*., 2007). Scully *et al*. (1988) reported that the ability of DS to accelerate AT-factor Xa interaction or HCII-T interaction is considerably increased in oversulfated naturally occurring forms and concluded that the possession of high charge density is not the only requirement for the interactions, and the spatial position of the sulfates are also of importance. With this, Pavao *et al*. (1998) have demonstrated that 4-*O*-sulfation of the galactosamine unit is essential for the anticoagulant activity of natural DSs isolated from marine invertebrates. Andoniades *et al*. (2002) have demonstrated that the specific activity of the DS of high purity

increased three times after a resulfation under mild conditions. All these results suggest that alterations in the sulfation pattern of the GAGs, and particularly DS, could be an important factor for the efficient control of the thrombogenic process and the multifactorial thrombotic events (Calabrese *et al*., 2004).

Inflammation is considered to be an important component of tumorigenesis, although the underlying mechanisms remain largely unknown (Coussens and Werb, 2002). Interestingly, benign tumor cells induce inflammatory response in the host and collaborate in establishing the tumor through a process called desmoplasia (Mareel and Leroy, 2003). Essentially, all of the elements that constitute the inflammatory response participate in the host reaction, which could, therefore, have an atrophic purpose for tumor cells (Arias *et al*., 2005). Nuclear factor κB (NF-κB) is a ubiquitous and well-characterized protein responsible for the regulation of complex phenomena, with a pivotal role in controlling cell signaling in the body under certain conditions. Among other functions, NF-κB controls the expression of genes encoding the proinflammatory cytokines, for example, interleukin-1 (IL-1), tumor necrosis factor-R (TNF-R), chemokines such as IL-8, macrophage inflammatory protein-1R (MIP-1R), and monocyte chemoattractant protein-1 (MCP-1), adhesion molecules such as vascular cell adhesion molecule (VCAM), and inducible enzymes such as cyclooxygenase-2 (COX-2), all of which play critical roles in controlling inflammatory processes (Aggarwal, 2004). CS is a GAG, which is naturally present in the extracellular matrix of articular cartilage (Fioravanti and Collodel, 2006). Recently, several studies indicated that CS played an important role in anti-inflammation and anticancer activities, and a few studies were performed to elucidate the underlying molecular mechanism. Legendre *et al*. (2008) reported that CS inhibited proinflammatory gene expression such as COX-2 and iNOS in chondrocytes; however, they did not provide detailed information of how CS suppresses the proinflammatory factor expression. Recently, Campo *et al*. (2008) found that CS inhibited collagen-induced NF-κB activation. However, the precise mechanism of CS was not investigated thoroughly either. Xu *et al*. (2008a) investigated the anti-inflammatory effects of CS on TPA-induced inflammation in mouse skin *in vivo* and explored the underlying molecular mechanism (Fig. 2.2). The results showed that CS suppressed TPA-induced edema, expression of cyclooxygenase-2, vascular cell adhesion molecule-1, and Akt signaling in the mouse skin. Therefore, it may be a promising strategy to inhibit the NF-κB activation in order to reduce the tumor formation. In addition, CS inhibited TNF-α-induced NF-κB activation and subsequent vascular cell adhesion molecule and inducible nitric oxide synthase expressions by blocking Akt signals in JB6 cells (Xu *et al*., 2008b).

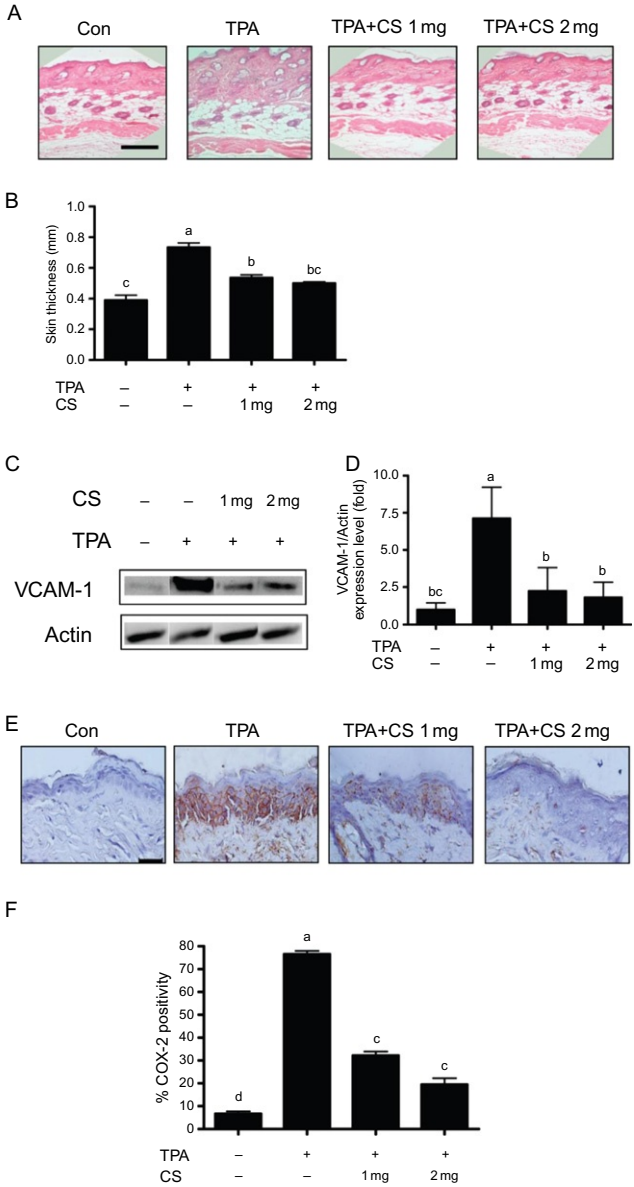


FIGURE 2.2 Inhibitory effects of CS on TPA-induced edema and expression of VCAM-1 and COX-2 in mouse skin. Mice were treated topically with TPA (200 μL of 10 nmol of TPA dissolved in acetone) in the presence or absence of CS (1 or 2 mg). Control animals were treated with acetone alone. (A) Skin section was stained with hematoxylin and eosin (original magnification, ×100; bar, 100 μm); (B) thickness of the skin (the thickness of the ear of each mouse was determined by averaging the values measured at five independent

C. Sea urchins

Astragalus membranaceus (Fisch) Bge. var. *mongolicus* (Bge.) Hsiao, a specialty crop in China, has been used in preparing functional foods such as soups and teas. It has been recognized for potential detoxification, diuresis, antiperspirant, myogenic, and anti-aging effects (Qi *et al*., 2006; Wang *et al*., 2006). Astragalus polysaccharides (APS) are considered as a group of possible bioactive components contributing to the beneficial effects of Astragalus. Several extractions of APS have been prepared and investigated for their monosaccharide compositions and molecular weights since the first one was purified from the roots of *A. membranaceus* in the 1980s (Fang and Wagner, 1988; Fang *et al*., 1982; Li *et al*., 2009; Masashi *et al*., 1992). The APS was extracted and purified from the roots of *A. membranaceus* and characterized for its chemical structure and potential health properties. The APS was composed of α-D-glucose residues with the estimated equivalent dextran molecular weight of 2.07×10^4 Da. Periodate oxidation analysis, 1D and 2D NMR spectroscopy demonstrated that APS has repeating (1→4)-linked backbone with a (1→6)-linked branch every 10 residues. The APS possess scavenging activities against hydroxyl radicals and hydrogen peroxide and showed chelating effect on ferrous ions. The APS was also able to bind cholic and chenodeoxycholic acids *in vitro*. In addition, APS was able to stimulate activity of purified mouse B cells without promoting T cell proliferation. These data provided information for future development of APS as a nutraceutical (Niu *et al*., 2011).

Malignancy of tumor and cancer cells is one of the most serious problems faced by cancer patients. This accounts for the eventual 12% of all deaths worldwide according to estimates by the World Health Organization (WHO; Ferlay *et al*., 2001). Hepatocarcinoma is a kind of tumor with both a high incidence and lethality rate. The inhibitory effect and evaluation of immunological mechanisms of a polysaccharide that was isolated from *Strongylocentrotus nudus* eggs (SEP) against hepatocellular carcinoma in H22-bearing mice were performed by determining its

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regions of the cross section); (C) Western blot analysis of VCAM-1 expression in mouse skin; (D) bands of interests were further analyzed by densitometer;
(E) immunohistochemical measurement of COX-2 in the mouse skin (dark brown color indicates COX-2 expression; original magnification, ×200; bar, 100 μm); (F) comparison of COX-2 labeling index in the mouse skin (COX-2-positive staining was determined by counting five randomly chosen fields per section, determining the percentage of DAB--positive cells per 100 cells at ×400 magnification). Different letters (a–c) denote statistical difference ($P<0.05$). Each bar represents the mean±SE ($n=3$). CON, control; TPA, TPA-treated group; TPA+CS, TPA-treated mice in the presence of CS (1 or 2 mg). Adapted from Xu *et al*. (2008a). With permission from ACS Publisher.

effects on the growth of transplanted tumors and immune response in H22-bearing mice. ICR mice inoculated with mouse hepatoma carcinoma cell lines H22 were treated with SEP at doses of 4, 8, 16mg/kg/day for 12 days. The effects of SEP were measured via the growth of the transplanted tumors, splenocyte proliferation, T lymphocyte counts, CTL activity, and the production of cytokines from splenocytes and the levels of serum Ig in tumor-bearing mice. In addition, the effects of SEP on Erk phosphorylation in mouse splenocytes and on the transcriptional activity of NFAT in Jurkat T cells were also investigated. Wang *et al*. (2011) showed that SEP significantly inhibited the growth of transplanted tumors in mice. SEP could not only remarkably enhance splenocyte proliferation, $CD4^+$ and $CD8^+$ T cell numbers as well as CTL activity, but it also elevated IL-2 and TNF-α secretion as well as IgA, IgM, and IgG levels in the serum. Further, the activation of Erk phosphorylation and the NFAT promoter by SEP promoted the transcription and expression of downstream gene IL-2. In conclusion, our study demonstrates that SEP effectively inhibits hepatocellular carcinoma *in vivo* via enhancement of host immune system function, and it could be a potential therapeutic drug for hepatocarcinoma.

D. Nudibranchs

Nudibranchs are some of the most beautiful animals found in the oceans. Their delicate bodies are usually brightly colored, often in striking multihued geometric patterns (Behrens, 1980). The hard shell used by most of their molluscan relatives for protection, which leaves their sensory rhinophores and oxygen-gathering branchial plumes decoratively exposed on their dorsums are absent in nudibranchs. They have large feet that provide locomotion, but only at speeds that are slow relative to potential predators. Nudibranchs tend to be found in shallow water habitats, where they frequently sit out in the open, blatantly exposing their vulnerability. Despite their apparent lack of physical attributes and behavioral patterns suited for defensive purposes, nudibranchs have few documented predators (Edmunds, 1968; Thompson 1960a,b). Astute field observations and some simple antifeedant experiments led marine biologists to propose that chemicals provide an invisible protective armor for these soft-bodied molluscs. In book ''*Between Pacific Tides*'' (Ricketts *et al*., 1968), the naturalist Ricketts wrote that ''Many nudibranchs, but especially the dorids, have a penetrating fruity odor that is pleasant when mild but nauseating when concentrated. Undoubtedly, this odor is one of the reasons why nudibranchs seem to be left strictly alone by their predatory animals.''

Mucus functions in many invertebrate physiological processes and also influences the structuring of the community and the ecosystem. Molluscan

mucus is mostly water. The remaining components are proteins, carbohydrates, and lipids. The predominant carbohydrates in the GAG fraction of *Arion ater* mucus were identified as glucosamine, iduronic acid, and galactose. The pattern of digestion of *A. ater* mucus with a series of polysaccharide lyases was consistent with its GAG fraction having the structure $(\beta GlcA1\text{-}4\alpha GlcN1\text{-}4\alpha IdA1\text{-}4\alpha GlcN1\text{-}4)_n$. Some GAG chains appeared to be linked to protein through GalN and/or GlcN, which would constitute novel protein–saccharide linkages, but others were linked through another, as yet unidentified, sugar (not xylitol or mannitol; Cottrell *et al.*, 1994). Mucus is probably released in dehydrated form in distinct, membrane-bound packages, which then absorbs water. Functional mucus is probably formed by mixing of mucins from different types of glands. Under small deformities, hydrated mucus is a viscoelastic solid, able to function as a rope. As stress increases, it yields to become a liquid which can return to the solid state once the stress is released. It is these properties that allow the locomotion in molluscs on what is seemingly called as an adhesion. On dehydration, the strength and stiffness of mucus production have been studied quantitatively by various methods, some gravimetric and some calorimetric using pedal, fecal, pseudofecal, and hypobranchial mucus: there are much spatial and temporal variations, though (Davies and Hawkins, 1998).

Mucus or slime is a gel exuded from the internal and external surfaces of many animals, both vertebrates and invertebrates. It is primarily composed of protein–sugar conjugates (glycoproteins) and water, although it may contain a wide range of dissolved materials such as salts, enzymes, antibodies, antibiotics, and various components of the cells which produce it. Its primary roles are surface lubrication and protection, although it is also used for capturing food particles, as defense against predators, and resistance to desiccation, among of its other functions (Eylers, 2008). Mucus plays a vital role in feeding. In filter-feeding bivalves, mucus aids the transport of food from gill to mouth and is employed to cleanse the mantle cavity of particles rejected by the labial palps. In gastropods, mucus nets and bags are used to trap food prior to ingestion and some groups roll their prey in mucus to prevent its escape. Pedal mucus may be ingested after it has become studded with organic material and perhaps act as a fertilizer for microbial growth (Davies *et al.*, 1990). A copious secretion of epithelial mucus is used to isolate molluscs from their environment, and mucus may also serve as an ionoregulator. Mucus may also contain specific products to render the animal poisonous, distasteful, or irritating. Agglutinin and lysozyme have been found in mucus from marine molluscs. Mucus secretion can present a considerable drain of energy (up to 70% of consumed energy). The fate of molluscan mucus is largely unknown and probably makes a considerable contribution to

POM in inshore waters, although it is readily degradable by marine microbes. Given the persistence of mucus, densities of benthic gastropods and their motility patterns, much of the gastropod-inhabited benthos is likely to be covered for most of the time with a layer of pedal mucus (Davies *et al*., 1990).

Invertebrates that lack GAGs synthesize sulfated polysaccharides such as spirulan from bivalve molluscs (Burson *et al*., 1956), horatin from annelids (Rahemtulla and Lovtrup, 1974), sulfated L-galactans from Styela (Pavao *et al*., 1994), and Kakelokelose, a sulfated mannan from another ascidian (Riccio *et al*., 1996). These polysaccharides, showing some structural features similar to GAGs, may well carry out the same or similar critical biological functions that GAGs do. Hovingh and Linker (1998) shown that CS is the major GAG present in the heart, mantle, and kidney of *Helix aspersa*. HS is present as a minor component mainly in heart and mantle. This HS differs somewhat from the major mammalian GAG, that is, the total sulfate content is lower and there is considerably less *N*-sulfated and more *N*-acetylated hexosamine. In *Aplysia*, ''heparin'' appeared to be the major GAG in the foregut and a minor component in the Ctenidium. The resistance of this heparin-like polymer to heparinase, however, indicates that it may be a highly sulfated HS or it may belong to the category of heparin–HS-like GAGs.

Mammalian mucus can exhibit abrupt stress softening (Celli *et al*., 2007), a rheological behavior attributed to the extended conformation, polyelectrolye nature, and liquid crystal propensity of its mucin molecules (Celli *et al*., 2007; Hyun *et al*., 2002). Slug mucus also exhibits this behavior. The slug mucus of *Lehmannia valentiana* does not contain mucins, but its Sm proteins contain A-domains, and A-domains are often found in systems that respond abruptly to hydrodynamic shear. Similarly, slug mucus exhibits a discontinuous response to shear stress. Below a critical strain ($\sim$0.15s), the mucus behaves as an elastic solid, but above this threshold, it transforms into a viscous liquid; uniquely, a short period of rest ($<$1s) is all that is required to return the hydrogel to its initial state (Cottrell *et al*., 1993; Denny, 1989; Ewoldt *et al*., 2007). The ability of their pedal mucus to undergo rapid and successive ''yield-heal'' cycles, and thereby act as a fluid ratchet, is what permits slugs and other gastropods to engage in adhesive locomotion (Denny, 1989; Ewoldt *et al*., 2007). Given the precedents above, it is tempting to suggest that a strain-dependent (and rapidly reversible) change in the conformation and binding properties of the A-domains in Sm-family proteins contributes to the distinctive rheology of slug mucus (Li and Graham, 2007). Moreover, GAGs are reported to be available in some kind of sea slugs (Fig. 2.3).

FIGURE 2.3 Glycosaminoglycans resources of some kinds of sea slugs. (A) *Melibe viridis*, (B) *Aplysia kurodai*, (C) *Glossodoris rufomarginata.* Adapted from http://www.umiushi.info/.

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