KYUNG-TAE KIM

SEASONAL VARIATION OF SEAWEED COMPONENTS AND NOVEL BIOLOGICAL FUNCTION OF FUCOIDAN EXTRACTED FROM BROWN ALGAE IN QUEBEC

Thèse présentée
à la Faculté des études supérieures et postdoctorales de l'Université Laval
dans le cadre du programme de doctorat en Sciences et Technologie des Aliments
pour l'obtention du grade de Philosophiae Doctor (Ph. D.)

DÉPARTEMENT DES SCIENCES DES ALIMENTS ET DE NUTRITION
FACULTÉ DES SCIENCES DE L'AGRICULTURE ET DE L'ALIMENTATION
UNIVERSITÉ LAVAL
QUÉBEC

2012

Résumé

Fucus vesiculosus et *Ascophyllum nodosum* sont des algues brunes comestibles et abondantes au Québec. Cependant, elles ont été négligées en raison de leur valeur potentielle inconnue et de la période de récolte limitée. Afin de valider leur utilité, la composition chimique de ces algues et l'activité inhibitrice des enzymes digestives de l'amidon par les fucoïdanes extraits de ces deux espèces d'algues ont été étudiés en fonction de la saison. Les composants principaux des algues sont dans l'ordre: les polysaccharides> minéraux> protéines> fucoïdane> lipides>> composés phénoliques, et leur quantité est très variable selon la période de récolte. *F. vesiculosus* contenait une plus grande quantité de protéines et de minéraux, alors que *A. nodosum* avait relativement plus de polysaccharides. Par conséquent, *F. vesiculosus* serait plus avantageux comme source d'éléments nutritifs. L'algue *A. nodosum* récoltée en Juillet a permis d'obtenir le fucoïdane ayant la pureté la plus élevée et le meilleur rendement. Les fucoïdanes extraits des deux espèces d'algues ont inhibé l'activité de l'α-glucosidase alors que seul celui extrait d'*A. nodosum* a pu, de plus, inhiber l'α-amylase. Le fucoïdane d'*A. nodosum* était un inhibiteur plus puissant que le fucoïdane de *F. vesiculosus* pour l'α-glucosidase avec des IC_{50} variant de 0,013 ~ 0,047 mg/ml, tout comme pour l'α-amylase avec des IC_{50} de 0,12 ~ 4,62 mg/mL selon le mois. Pour comprendre les facteurs clés expliquant les différences d'inhibition d'α-amylase entre les fucoïdane d'*A. nodosum* et *F. vesiculosus*, certaines caractéristiques structurales ont été analysées et comparées à du galactofucoidane qui a servi de contrôle. A partir des résultats obtenus, il est confirmé que la masse moléculaire plus faible (637 kDa) du fucoïdane d'*A. nodosum* et la présence de sulfates sont liées à son activité inhibitrice. Nous avons émis l'hypothèse que les faibles masses moléculaires permettent d'exposer facilement les groupements sulfate qui peuvent agir sur l'α-amylase par interaction électrostatique, et donc d'inhiber son activité. En conclusion, les algues brunes du Québec présentent un potentiel d'utilisation important pour leur valeur nutritionnelle et leurs composés bioactifs. Le fucoïdane a montré une activité d'inhibition des enzymes digestives de l'amidon (α-amylase et α-glucosidase) et cette activité est différente selon les espèces d'algues et la période de récolte. Une meilleure compréhension du mécanisme inhibiteur par le fucoïdane peut être utile afin de développer un ingrédient fonctionnel permettant de prévenir le diabète de Type-2.

Abstract

Fucus vesiculosus and *Ascophyllum nodosum* are edible brown seaweed and abundantly available in Quebec. However, they have been neglected because of their unknown value and technical limitation in harvest. In order to validate their usefulness, chemical composition in seaweeds and starch digestive enzyme inhibition activity by fucoidan extracted from two seaweed species were investigated with different seasons. The major components in both seaweeds were in order: polysaccharide > minerals > protein > fucoidan > lipid > phenol, and their quantity was quite variable depending on harvesting timing. *F. vesiculosus* contained larger amount of proteins and minerals, while *A. nodosum* had relatively more polysaccharides. Therefore, *F. vesiculosus* are advantageous as a nutritional source. Especially, from *A. nodosum* harvested in July, a fucoidan having higher purity and better yield was obtained. Fucoidans from two seaweeds species inhibited α-glucosidase activity while, only fucoidan from *A. nodosum* could inhibit α-amylase activity. *A. nodosum* fucoidan was a more potent inhibitor than *F. vesiculosus* fucoidan for α-glucosidase with IC_{50} of 0.013 ~ 0.047 mg/mL, and for α-amylase with IC_{50} of 0.12 ~ 4.62 mg/mL depending on harvest month. To understand the key factors explaining the difference in α-amylase inhibition between *A. nodosum* fucoidan and *F. vesiculosus* fucoidan, structural characteristic was analyzed and compared with galactofucoidan as a control. From the obtained results, it is confirmed that smaller molecular weight (637 kDa) of *A. nodosum* fucoidan and the presence of sulfates are related to its inhibitory activity. It is proposed that small molecular weight permits to expose easily sulfate groups for interaction with α-amylase throughout electrostatic interactions, and therefore inhibiting its activity. In conclusion, brown seaweeds in Quebec have a considerable importance for nutrition and bioactive products. Fucoidan shows the inhibition activity for starch digestive enzymes (α-amylase and α-glucosidase) and its activity is different depending on seaweed species and harvesting period. Further understanding of the inhibitory mechanism by fucoidan can be useful to develop a functional ingredient to help preventing for Type-2 diabetes.

Preface

This thesis has been prepared to present the results as scientific publications and is organized as follows.

The first chapter « Literature Review » contains the descriptions of general characteristic of seaweeds, structural characteristic and biological ability of fucoidan, and starch digestive enzyme.

The second chapter is entitled « Seasonal variation of fucoidan and the evaluation of nutritional components of brown algae in Quebec: *Fucus vesiculosus* and *Ascophyllum nodosum* ». This chapter shows the results on seasonal variation of chemical components of seaweeds (*F. vesiculosus* and *A. nodosum*) in Quebec and key characteristics of their fucoidan extracts. We observed the influence of harvesting month and seaweed species on the variation of components. Especially, the characteristic of fucoidan has been observed depending on seaweed harvesting period. I have done all the laboratory experiments, data analysis and manuscript preparation under the supervision of Prof. Sylvie L. Turgeon, my supervisor.

The third chapter deals with the topic, « Alpha-amylase and alpha-glucosidase inhibition is differentially modulated by fucoidan obtained from *Fucus vesiculosus* and *Ascophyllum nodosum* », and is also being prepared to publish. In this chapter the possibility of fucoidan as an inhibitor of starch digestive enzymes (Human salivary α-amylase and α-glucosidase) was evaluated possibly to control blood glucose level. I have done all the experimental work, analysis and preparation of the results. Ms Laurie-Eve Rioux assisted me for the statistical analysis and reviewing of the article. Professor Sylvie Turgeon supervised me for the experimental planning, interpretation and thorough correction of the manuscript.

The fourth chapter is titled as « Inhibition of alpha-amylase by fucoidan obtained from two species of brown seaweed: the effects of the structural characteristics ». This chapter was performed to find out a key factor of α-amylase inhibition of fucoidan by comparing structural characteristics of two fucoidans (*F. vesiculosus* and *A. nodosum*), and by using galactofucoidan from *Saccharina longicruris* as a positive control. The analysis on monosaccharide and linkage was done by Laurie-Eve Rioux Ph.D. who is a research associate at the department of Food

Science and Technology of Laval University. I performed fucoidan extraction, the other structural analysis and enzyme inhibition assay *in vitro*. I prepared the manuscript. Prof. Sylvie Turgeon supervised the activities and corrected the manuscript.

Remerciements

Tout d'abord, je voudrais exprimer toute ma gratitude et mes sincères remerciements à la Professeure Sylvie Turgeon. Depuis le début, elle m'a supporté et encouragé en me permettant de compléter mon projet de doctorat. Quand j'avais des problèmes professionnels mais aussi personnels associés à la vie à Québec, ses conseils m'ont été précieux. Son expression 'Penser positif' m'a été utile tout au long de mon doctorat. Elle a montré beaucoup de patience en m'écoutant et discutant avec moi malgré mon français laborieux. Ma directrice, Dr. Sylvie Turgeon, grâce à ses grandes connaissances scientifiques et ses grandes qualités humaines, m'a fourni d'excellentes conditions de travail qui m'ont permis d'obtenir de bons résultats de recherche. Je tiens à la remercier de m'avoir appris les principes essentiels en science que sont la curiosité et la rigueur scientifique, la ténacité, l'humilité et la patience.

Merci au Professeur François Castaigne d'avoir fait la prélecture de ma thèse et pour ses bons conseils afin de l'améliorer. Je ne vais pas oublier votre aide.

Je tiens aussi à remercier les membres du jury de ma thèse : le Professeur Paul Angers du département des Sciences des Aliments et de Nutrition de l'Université Laval et le Professeur Oon-Doo Baik du department of Chemical and Biological Engineering, de l'Université de la Saskatchewan. Merci de vos bons conseils pour améliorer la qualité de ma thèse.

Et plus, je tiens à remercier tout spécialement Madame Laurie-Eve Rioux. Elle m'a aidé dans mon travail au laboratoire depuis le début de ma thèse, ses conseils et avis ont été très appréciés. Elle m'a aussi aidé au cours de la rédaction et pour certaines analyses statistiques de ma thèse.

Je remercie aussi Mme Diane Gagnon, Mme Anne-Françoise Allain et M. Alain Gaudreau pour leur assistance technique et scientifique et les analyses au laboratoire. Ces aides ont été précieuses tout au long de ma thèse.

Je remercie le CORPAQ pour le financement de cette étude et ABK-Gaspésie Inc. pour la

récolte des algues. Et plus, je tiens à remercier le MAPAQ plus particulièrement M. Piotr Bryl pour la préparation des algues. En outre, j'ai apprécié le support de Mme. Laure Devine, Mme Caroline Lafleur et M. Denis Gilbert (Pêches et Océans Canada) pour les informations environnementales.

Je tiens à remercier les membres de notre équipe et mes amis Dr. Gérard Bédie, Dr. Sami Gaaloul. Je n'oublie pas dans mes remerciements Mme Johanne Gagnon qui est mon amie à Québec et qui m'a accueilli chez elle dans d'excellentes conditions pour la fin de ma thèse. Vous étiez mon autre mère au Canada.

Merci, Professeur Keun-Tae Lee qui a été le directeur de ma maitrise en Corée du Sud et m'a donné la chance d'aller étudier au doctorat à l'Université Laval.

Ce travail a été rendu possible grâce au sacrifice et la patience de ma famille en Corée du Sud. Mes parents et mes frères ont sacrifié beaucoup de choses pour mes études et ils ont attendu la fin de ma thèse très longtemps.

Je remercie mon amour la Docteure Hyun-Ju KIM. Elle m'a supporté continuellement s'occupant de ma santé et me donnant du courage. Elle m'a donné et m'a entouré de son amour même si j'étais très stressé. Grâce à son amour, j'ai pu compléter ma thèse.

List of figures

List of tables

Contents

General introduction

For several centuries, humans have used countless terrestrial species of plants as foods and as sources of numerous useful materials to produce functional foods, cosmetic products and medical substances. However, many of these resources are close to exhaustion or will run into limitations on their use because of the pressures of an ever-increasing population of consumers and changes in environmental conditions. Marine plants have also been valuable sources of useful materials and studies of algae or seaweed have been on the fast track. The study of marine algae has a shorter history than that of plants, and the objectives of research on algae differ considerably between Asia and the Occident, because of the different patterns of algae consumption. Seaweeds have been an important food source in Asia (especially Korea and Japan) since ancient civilization. In addition, seaweed is now quite expensive. Korea consumed an estimated 140 million tons of the red seaweed *Porphyra tenera* kjellman (laver) as food in 1997 (based on the report of Korea Maritime Institute, 1999), while Western countries like Canada and the USA use it in animal feeds.

Many now consider seaweed as a new natural resource from which many functional materials may be extracted to replace terrestrial resources used throughout the 20th century. In addition, seaweed is a more attractive marine resource than many others, since it contains many functional components. The functional components in seaweed have evolved as mechanisms of survival under severe conditions such as low temperature, physical impact of the action of waves, tides and inflow, sea salt, intense ultraviolet rays and dryness due to wind (Barsanti and Gualtieri, 2006; Percival and McDowell, 1967).

Among the three types of seaweed (brown, red and green), brown seaweed contains the most polysaccharide. The biological activities of the polysaccharides in brown seaweed have been described in the scientific literature (Rupérez et al., 2002; Koyanagi et al., 2003). Polysaccharides are classified in three groups: mucilaginous, storage and structural. Fucoidan, laminaran and alginic acid are the major polysaccharides of seaweeds. Over 50% of brown seaweed is composed of polysaccharides and much of them are sulfated, which is rarely observed in land plants (Cho et al., 1999).

Fucoidan was isolated and named by Kylin in 1913 (Percival and McDowell, 1967). It is an intracellular sulfated water-soluble and highly hygroscopic polysaccharide (Percival and McDowell, 1967). The principal function of fucoidan is to protect the seaweed surface from dryness and to

contribute to the formation of a gel network (Percival and McDowell, 1967). It is also known as a bio-functional component with anti-coagulant, anti-thrombin (Cho et al., 1999; Soeda et al., 2000), anti-HIV and anti-tumor activities (Moen and Clark, 1993; Béress et al., 1993). Toida et al. (2003) reported that the sulfate groups in the fucoidan molecule play a key role in the biological activities of fucoidan. Recently, new function of fucoidan from *Undaria pinnatifida* have been reported by Cho et al. (2011a) that was the inhibition of amylase activities (one of starch digestive enzymes) for limiting glucose absorption. The inhibition of starch digestive enzymes can prevent the increase of blood glucose level and thus it can be useful to control diabetic symptom.

It has long been presumed that the chemical composition of seaweed is the result of many factors such as harvesting region, season and seaweed species. The production of fucoidan in the plant is also subject to environmental conditions. Season is an especially significant factor. It is therefore important to understand the seasonal variations of seaweed components in order to determine the proper time to harvest seaweed for a given application. Many studies have examined the relationship between season and seaweed components (Chapman and Craigie, 1977; Kim et al., 1996; Fleurence, 1999; Rupérez et al., 2002). Winter to spring, the proliferation period of seaweed has been suggested as the optimal harvesting period (Chapman and Craigie, 1977 & 1978; Kim et al., 1996).

There are three major species of brown seaweed in the lower St. Lawrence River in Quebec. These are *Fucus vesiculosus*, *Ascophyllum nodosum* and *Laminaria longicruris*. The world rate of consumption of brown seaweed is higher than for other species and the amount of seaweed harvesting is increasing every year (Barsanti and Gualtieri, 2006). However, seaweed harvesting from winter to spring is not profitable in Quebec, since the St. Lawrence River is covered with thick ice during the winter and often into the spring as well. The study of seasonal variations of seaweed components is therefore especially important for the use of brown seaweed harvested in Quebec. The relationship between season and components has been studied in the case of *L. longicruris* (Anderson et al., 1981; Souchet, 2004), but not for *F. vesiculosus* or *A. nodosum*. There is thus a lack of information on the variation of the chemical components of seaweed for efficient utilization of seaweed harvested in Quebec.

In the present study, the seasonal variation of the overall chemical composition of *F. vesiculosus* and *A. nodosum* in Quebec was evaluated from spring to autumn for three years (2002, 2003 and 2005).

The fucoidan and polyphenol contents, two seaweed components associated with biological activity, were then studied. Also, the purity and quantity of fucoidan were evaluated according to the season. The possibility of using fucoidan as an inhibitor of starch digestive enzymes (α-amylase and α-glucosidase) to control the symptoms of type-2 diabetes was then investigated as a function of seaweed species and harvesting period. And finally, the dependence of the amylase-inhibiting function of fucoidan on seaweed species was studied and an attempt was made to determine the nature of the key factor underlying the different biological activities of fucoidans.

The information obtained from this study should be useful for determining the best period for harvesting seaweeds, for using brown seaweed as a source of food and ingredients such as fucoidan to control starch digestive enzymes (α-amylase and α-glucosidase).

Chapter 1. Literature Review

1.1 Marine algae (seaweed)

Algae are autotrophic, aerobic organisms that contain chlorophyll and photosynthesize like vascular plants. They grow in water or on shorelines and "seaweed" is a common name for marine algae (Cambridge dictionaries. 2000). Marine algae contain unusual components compared to plants as a result of adaptation to the severe conditions under which they grow. For example, the floating structures of *Fucus vesiculosus* and *Ascophyllum nodosum* are mostly exposed to the air while the "root" portion and much of the stalk are submerged in seawater. The portion that is exposed to dry conditions also varies with tidal activity. In addition, much of the deeply immersed structure does not receive enough sunlight for photosynthesis. The components that enable marine algae to survive vary depending on species. For example, fucoidan is present only in brown algae and not in red or green algae. The taxonomical classification of seaweed is now based on Kingdom > Division > Class (Barsanti and Gualtieri, 2006) but seaweed in general is commonly identified by color since this is convenient. Green, red and brown seaweeds are thus recognized (Tilden, 1935).

1.2 Utilization of marine algae

The uses of seaweed differ greatly between Asia and western nations. In Asia, seaweed is a common food and part of the food culture. For example, *Undaria pinnatifida* (a brown alga) is prepared as a soup for consumption by parturient women in Korea. Laver (called *kim* in Korea), a mixture of red seaweeds such as *Porphyra yezoensis* and *Porphyra tenera*, is a highly appreciated ingredient in *maki*. At least fourteen species of laver are used in Korea and Japan (Lee and Ahn, 1986).

In comparison, seaweed is used in western society mostly as raw material for producing commodity products, which may be used as food ingredients but are more often used for industrial purposes. The brown seaweed *Ascophyllum nodosum* is used on a commercial scale in North America in plant fertilizers and has been studied as an additive in pig feed by Archer et al. (2008) and Gardiner et al. (2008).

The study of marine algae has so far focused primarily on its use as bio-fuel (micro-algae) or as a source of health products or for use in cosmetics and so on. While cornstarch is currently the

principal feedstock for bio-fuel production, cellulose from seaweed would have the advantage of providing bio-fuel without competing for land use or pushing corn prices upward. The study of both macro-algae and micro-algae for bio-fuel production is increasing due to the large amounts of polysaccharide and cellulose that these plants contain (Hossain et al., 2008; Wi et al., 2009).

1.3 Components of marine algae and their potential

1.3.1 Protein/nitrogen

Protein/nitrogen content is important for human nutrition and represents an essential element for plant fertilizers (Stewart, 1974). The protein (nitrogen) content of marine algae increases their value both as food and as fertilizer for plant cultivation. Numerous studies of protein content have been done to increase the value of marine algae.

The protein content of dried marine algae is typically 10-30%. It is 15-25% in green seaweed, 5-15% in brown seaweed and 15-30% in red seaweed (Table 1-1). The protein content of marine algae is relatively constant and high compared to plants (Souchet, 2004; Fleurence, 1999).

Table 1-1. Protein in various marine algae used in the food industry

Species	Protein content (% of dry mass)
Palmaria palmata	8-35
Porphya tenera	33-47
Ulva lactuca	10-21
Ulva pertusa	20-26
Laminaria digitata	8-15
Fucus species	3-11
Ascophyllum nodosum	3-15

(Reference; Fleurence, 1999)

Marine algae synthesize protein through fixation of atmospheric nitrogen and protein content therefore varies with harvesting period. Environmental factors such as light, temperature and salinity influence protein synthesis (Stewart, 1974; Barsanti and Gualtieri, 2006). Chapman & Craigie (1977) and Souchet (2004) reported the correlation between protein and water nitrogen content and as high temperatures decrease dissolved nitrogen content in seawater, consequently nitrogen fixation is reduced and the marine algae's protein content harvested in the summer. In addition, higher temperatures also increase energy consumption due to algal respiration (Anderson et al., 1981). This energy demand exceeds the rate of energy capture by photosynthesis and results in the diversion of fixed and stored carbon away from nitrogen assimilation (Graham and Wilcox, 2000; Anderson et al., 1981). Other reports (Germann et al., 1987; Chapman & Craigie, 1977) confirm that protein content is relatively low in summer and higher in winter.

1.3.2 Lipids

The lipid content of marine algae is usually low and generally less than 4% of the dried mass, although *Sargassum kjellmaniamum* (a brown alga) contains more than 6% (Sánchez-Machado et al., 2004). However, the relationship between lipid content and species has been difficult to determine with precision (Ito and Tsuchiya, 1977). In brown algae, the fatty acids include primarily C16, C18 and C20 forms, with palmitic acid (C16) making up 10-15% and considered as a major fatty acid. The fatty acids composition in seaweed has been linked to decreased risk of heart disease, thrombosis and atherosclerosis and to antiviral activity (Sánchez-Machado et al., 2004). In addition, marine algal lipid contains antioxidants such as tocopherol, which can be used in cosmetics or health products (Norziah and Chio, 2000; Dawczynski et al., 2007).

1.3.3 Minerals

Mineral content varies with algal species (Table 1-2), growing region, season and environmental conditions. Algae absorb and store several minerals from seawater. Minerals generally represent 10-35% of the dry mass of seaweed (Ito and Hori, 1989) as compared to 5-10% of the dry mass of land vegetation (Rupérez, 2002). Brown algae are rich in nutrients such as calcium, magnesium, potassium, sodium, phosphorus, sulfur, iodine, iron and so on. The iodine content of brown algae is

especially high. Iodine concentration varies depending on seaweed species (Küpper et al. 1998; van Netten et al., 2000; Hou and Yan, 1998; Chance et al. 2009). Mineral products made from marine algae are manufactured and marketed like certain vitamin products as natural health products.

Table 1-2. Ash contents of edible brown and red algae

(%, dry basis)

Type	Genus	Ash *
Brown algae	*Fucus*	30.10±0.20
	Laminaria	35.59±0.40
	Wakam	29.26±0.24
Red algae	*Chondrus*	21.08±0.12
	Nori	20.59±0.16

*Analysis condition: 550℃ for 16h (reference; Rupérez, 2002)

1.3.4 Polysaccharide

Polysaccharides are polymers of monosaccharides and major components of marine algae representing over 60% of dry weight (Marinho-Soriano et al. 2006; Rioux et al, 2007a). Each polysaccharide has specific chemical characteristics such as molecular mass, degree of polymerization, degree of branching, types of monosaccharide units and types of linkage. These characteristics vary among polysaccharides within and between algal species. Furthermore, some monosaccharides can bear substituents as sulfate which have an important effect on functionality. For example, the presence, position and type of sulfate groups determine the physicochemical properties of polysaccharides such as fucoidan (Rioux 2005).

Marine algae contain large amounts of non-starch polysaccharides that cannot be digested completely by the human digestive system and which therefore have potential as new sources of dietary fiber, prebiotics or other functional ingredients (Lahaye, 1991; Mabeau and Fleurence, 1993). As with plant fiber from other sources, seaweed fiber is interesting because its consumption has been associated with a significant reduction of chronic diseases such as diabetes, obesity, heart diseases, cancer and so on. It has been shown that the physiological effect of fiber is related to its physicochemical properties. Fiber is classified as soluble and insoluble fractions (Roehring, 1988). Soluble fiber can

slow down digestion and absorption of nutrients by increasing viscosity and might thereby decrease blood sugar and cholesterol. In contrast, insoluble fiber decreases intestinal retention time and fecal mass (Mabeau and Fleurence, 1993).

Algal polysaccharides are divided into three groups based on their role: structural polysaccharide, intercellular mucilage and storage polysaccharide. Polysaccharide composition differs widely among green, brown and red algae and marine algal polysaccharides as a whole offer a wide range of commercially valuable physicochemical properties for the food, pharmaceutical, cosmetics and other industries.

1.3.4.1 Polysaccharides in green marine algae

Like vascular plants, green seaweed contains cellulose as the principal structural polysaccharide (Yaich et al. 2011). However, some green seaweed contains structural polysaccharides other than cellulose. For instance, *Codium* uses β-1,4-mannan as a structural polysaccharide (Percival and McDowell, 1967). Intercellular mucilage polysaccharides are glucuronoxylorhamnans, glucuronoxylorhamnogalactans and xyloarabinogalactans, which bear sulfate groups. Amylose and amylopectin are stored as energy sources (Percival and McDowell, 1967).

1.3.4.2 Polysaccharide in brown marine algae

Cellulose is present in brown seaweed as structural polysaccharide and its content range from 5.7 to 14% (Park et al. 2000). The mucilage polysaccharides are alginate, fucoidan and laminaran. Alginate was first separated in 1883 by Standford in England (Park et al., 1997b; Percival and McDowell, 1967) and was identified as an intercellular or cell wall matrix. As shown in Table 1-3, alginate generally makes up to 10-30% of the dry mass of marine algae (Park et al., 2000). Alginate is a β-1,4-linked polymer of D-mannuronic acid (M) and L-guluronic acid (G). Its properties including solubility depend on the M/G ratio and on the molecular mass, which ranges from 150 to 1700 kDa, depending on the source and the extraction method (Moe et al., 1995). In general, the molecular mass of alginate for commercial use ranges from 32 to 200 kDa (Park et al., 2000). Alginate is highly appreciated in the food industry for its gelling, thickening, emulsifying, and stabilizing properties (Park et al., 1997b).

Fucoidan is a well-known mucilage heteropolysaccharide in brown algae (Park et al., 1997a; Park et al., 2000; Percival and McDowell, 1967). It is synthesized in the Golgi apparatus inside cells and is found in the intercellular spaces throughout the algal tissue (Park et al., 1997a). The principal function of fucoidan is to prevent drying out of the plant exposed to the air by tides (Percival and McDowell, 1967). The fucoidan content of brown algae varies with species, growing region and season (Park et al., 2000). More information on fucoidan is provided in section 1.6.

Laminaran is a β-glucan with a molecular mass of 3.5~5.3 kDa (Rioux, 2005; Souchet, 2004). It is the main storage polysaccharide in brown algae. Its known biological activities include stimulation of the immune system and cytotoxic effects on tumor cells (Nagaoka et al., 2000). Laminaran is a β-1,3-linked polymer of mostly D-glucose with some β-1,6 linkage (Park et al., 1997a and 2000). The detailed structure of laminaran varies among species and its solubility depends on the amount of β-1,6 linkage. Laminaran is classified as soluble or insoluble in cold water (20°C), and the insoluble laminaran fraction is solubilized in water at 60-70°C (Souchet, 2004; Park et al. 2000). Its solubility depends on chain length (CL) and the degree of polymerization (DP). For soluble laminaran, CL = 7-11 and DP = 26-31, while for insoluble laminaran, CL = 15-19 and DP = 16-24 (Souchet, 2004; Nelson and Lewis, 1974).

Table 1-3. Polysaccharide content of brown algae

(%, dry basis)

Species	Laminaran	Alginate	Crude fiber
Laminaria japonica	1.3	22.5	9.1
L. angustata var. longissima	1.4	27.2	12.8
Kjellmaniella gyrate	4.3	30.2	16.1
Undaria pinnatifida	0.6	22.7	15.2
Anthrothamnus bifidus	0.7	16.6	6.8
Elisenia bicyclis	13.3	17.9	7.1
Ecklonia kurome	-	16.0	4.6
Pelvetia wrightii	1.6	25.0	17.1

(Referred source; Translated from Park et al., 2000)

1.3.4.3 Polysaccharides in red algae

Most red algae contain cellulose as the main structural polysaccharide (Park et al., 2000), although species of *Rhodeminea* contain β-1,3 and β-1,4 xylans for this purpose (Park et al., 1997a). The mucilage polysaccharide of red algae is α-1,3-linked and β-1,4-linked sulfated galactan. Other major polysaccharides in red algae are agar, carrageenan and porphyran (Park et al., 2000).

Agar is a mucilage polysaccharide used widely for commercial purposes as a gelling agent in foods and in media for culturing microbial organisms (Fuse and Goto, 1971). Agar is composed of agarose (70%) and agaropectin (30%). Agarose contains 1,4-linked α-3,6-anhydro-L-galactose (about 34%) and 1,3-linked β-D-galactose (about 56%). Agaropectin is essentially agarose with sulfate ester and D-glucuronic acid side groups (Park et al., 1997a; Park et al., 2000). Properties of agar such as gel forming ability or gel strength depend on the ratio of agarose to agaropectin, the sulfate content, harvesting region and season and the extraction method (Fuse and Goto, 1971; Park et al., 1997a; Park et al., 2000).

Carrageenan is another sulfate-containing polysaccharide abundant in red algae (Park et al., 1997a; Park et al., 2000). It consists of D-galactose and 3,6-anhydro-D-galactose sulfate esters. The gelling ability of carrageenan is lower, while its viscosity is higher than that of agar (Park et al., 1997a). According to Campo et al. (2009), carrageenans are widely used in the food industry because of their physical properties such as thickening, gelling and stabilizing abilities. These properties are useful to

control the texture and viscosity of dairy products, and are utilized for binders and stabilizers in the meat-processing industry such as sausages and low-fat hamburgers.

1.3.5 Polyphenols/phenolic compounds

Phenolic compounds encompass a wide range of molecules. Benzene rings bearing a hydroxyl group are antioxidative site. Flavonoids are a group of polyphenols (Boudet, 2007). Holiman et al. (1996) reported that over 4,000 different flavonoids have been observed, and that at least a thousand kinds of phenolic compounds undiscovered could exist. However, relatively few phenolic compounds have been identified and studied completely. These include primarily flavonones, flavonols, anthocyanin, tannins and so on (Fig. 1-1).

The phenolic compounds which are discovered in marine algae have strong antioxidant activity (Jiménez-Escrig et al., 2001). Some of these compounds also displayed detoxification ability due to their chelating capability (Ragan et al., 1980). For instance, various biological activities such as anticancer and anti-proliferation have been reported for phenolic compounds. The activities depend on the antioxidant and/or chelating abilities of polyphenols. For example, flavonoids exert these effects through antioxidant properties, since oxygen free radicals are related to cancer and inflammation (Holiman et al., 1996).

In addition, phenolic compounds strongly bind to proteins and can inhibit enzymatic activity. Tannin from the brown seaweed *Ascophyllum nodosum* also displays several biological activities (Wang et al., 2008). In addition, numerous reports on the phenolic extracts from seaweed have mentioned α-amylase or α-glucosidase inhibition (Zhang et al., 2007; Ohta et al., 2002; Bhandari et al., 2008). The interactions between protein and polyphenols will be described in greater detail below (section 1.8.1.1.2.).

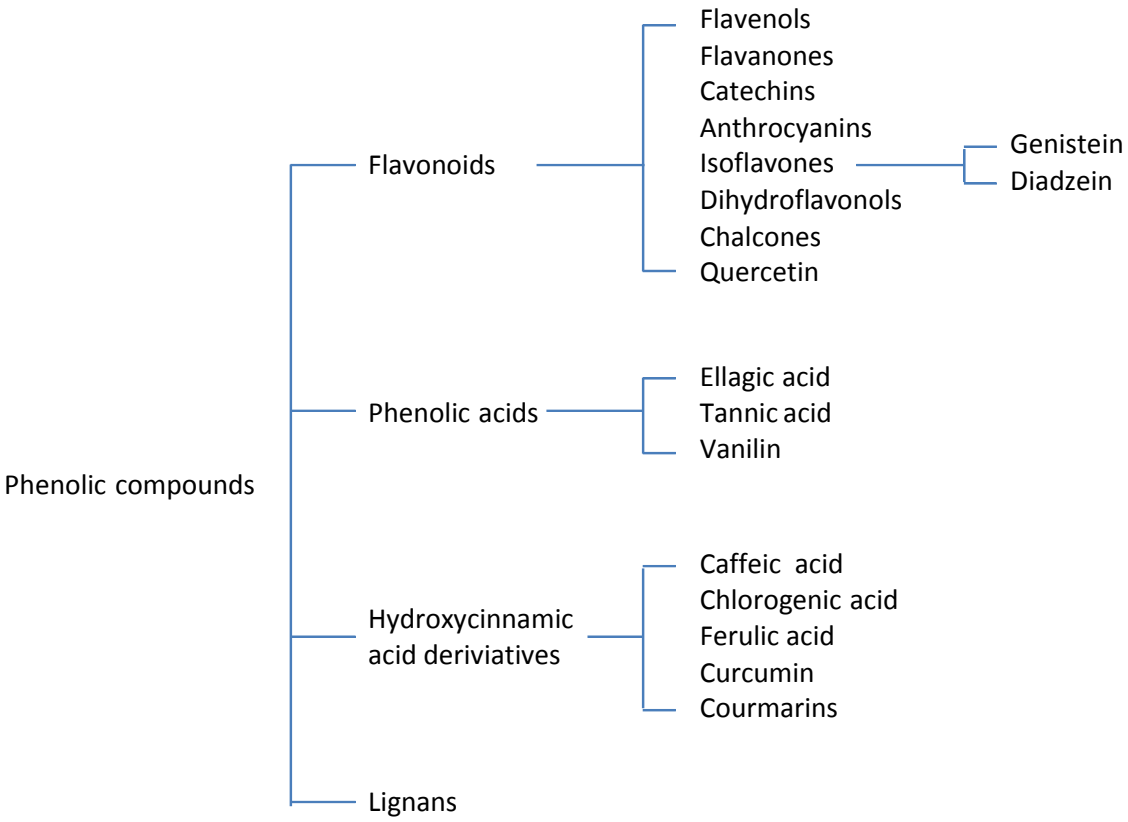

Figure 1-1. Nomenclature of some of phenolic compounds from natural sources

1.3.6 Vitamins

Like many vegetables, marine algae contain several vitamins, their composition in several algae is shown in Table 1-4. The particularity of marine algae is the presence of vitamin B_{12} (cyanocobalamin), which is rare in vegetables. Vitamin B_{12} has long been considered an animal protein factor but was found recently in marine seaweeds (Park et al, 1997a and 2000). The vitamin B_{12} in marine algae is similar to that formed in animal intestines and can be used to replace the animal sources. Marine algae are also sources of vitamin E (α-tocopherol) and generally contain this vitamin at concentrations of 0.7-8 mg per 100 g. However, the vitamin E content is variable according to the season. In the case of vitamin C, the period from spring to early summer produces algae with the highest content (Park et al., 1997a).

Table 1-4. Vitamins contents (mg/100g dry basis) of marine algae

Type of algae	A	B1	B2	B6	B12	C
Green						
Ulva pertusa	590	0.09	0.28	nd	0.0063	27-41
Monostroma nitidum	2647	0.12	0.85	nd	0.0013	75-80
Enteromorpha linza	12495	0.15	0.12	nd	0.0098	10-257
Brown						
Laminaria spp.	619	0.09	0.02	0.03	0.0003	3-91
Ecklonia cava		0.11	0.25	nd	0.0003	
Hizikia fusiforme	359	0.03	0.27	nd	0.0006	0-92
Undaria pinnatifida	813	0.73	1.88	nd	nd	156
Red						
Porphyra tenera	15748	0.17	2.31	1.04	0.0291	10-831
Gelidium amansii	5329	0.16	1.80	nd	0.0036	nd

nd; not determined. (Reference; Translated from Park et al., 1997a)

1.4 Environmental effects on the growth of marine algae

Marine algae contain the six principal elements of biomass, namely carbon, oxygen, hydrogen, nitrogen, phosphorus and sulfur (Park et al., 1997a; Park et al., 2000) and several minor elements. Smaller quantities of calcium, potassium, sodium, chloride, magnesium, iron and silicate are required for the metabolism of seaweed. Environmental conditions (light, temperature) and nutrient availability (nitrogen, minerals, silicate, phosphorus, etc.) could have an effect on seaweed growth. Algae utilize nitrogen in water to synthesize protein, and phosphorus is needed for the production of nucleic acids and ATP for energetic functions. Barsanti and Gualtieri (2006) reported the importance of phosphorus as it is connected mainly to the growth of algae and plants. If all phosphorus is consumed, autotroph growth will cease, no matter how much nitrogen is available. Furthermore, the sporulation of *Rhodochorton spp.*, a red seaweed, is enhanced at low salinity (Round, 1981).

Each factor is important in view of Liebig's Law (Barsanti and Gualtieri, 2006). The concept of Liebig is the "Law of the Minimum", meaning that algal growth is not limited by the total amount of nutrients available but by the nutrient available in the smallest quantity relative to its requirement. Concentration will be an indicator of limitation of a specific nutrient, however, and the rate of supply of that nutrient or its turnover time is more important in determining the degree of limitation (Barsanti and Gualtieri, 2006).

Marine algae contain special components not found in terrestrial plants, because of the severe conditions of their environment. Algae could live completely immerged in water or at the surface and could then be immerged or exposed to air with tides. The root of the alga is fixed to a submerged solid surface and much of the plant is submerged. In contrast, the extremity of the alga floats on the water and is dried by sunlight and dry air. The length of the exposed portion of the alga is dependent on tides and changes frequently. Parts that are in deep water do not receive sunlight for photosynthesis. In order to survive under the worst conditions, algae contain some unique components not discovered in plants and these components differ according to algal species (Amer et al., 1997). For example, fucoidan is detected only in brown algae but not in red and green algae.

Numerous species of algae grow in the Eastern part of Canada, and among them *Fucus vesiculosus* and *Ascophyllum nodosum* exist in quantities to permit a commercial utilization.

1.5 *Fucus vesiculosus* and *Ascophyllum nodosum*

1.5.1 Characteristics of *F. vesiculosus* and *A. nodosum*

Fucus vesiculosus and *Ascophyllum nodosum* are assorted to the class of *Phaeophyceae* in the scientific classification. They are very well known brown seaweeds and they live completely submerged as an intertidal marine alga (Round, 1981). These species are useful as sources of bioactive elements. The common or commercial name of *Fucus vesiculosus* is bladderwrack, a type of seaweed that grows on and adheres to stones (Dickinson, 1963). Bladderwrack is found on the coasts of the North Sea, the western Baltic Sea and the Atlantic and Pacific Oceans. It is a common food in Japan and is used as an additive or flavoring agent in various food products in Europe. Recently, fucoidan extracted from *Fucus vesiculosus* gained interest because of its biological activities and potential medical applications. More discussion of the biological activities of *Fucus vesiculosus* will be provided in section 1.6.6.

Ascophyllum nodosum is commonly known as knotted sea-wrack. It alternates between diploid and gametophyte life stages. Water temperature at high tide has been associated with *Ascophyllum nodosum* gamete release (Bacon and Vadas, 1991). The onset, midpoint, and end stages of gamete release occur at 6, 10, and 15°C respectively (Bacon and Vadas, 1991). The general composition of *A. nodosum* and *F. vesiculosus* was studied by Rioux (2005) and is presented in Table 1-5.

Table 1-5. Chemical components of *F. vesiculosus* and *A. nodosum*

(%, dry basis)

Species	Ash	Protein	Lipid	Polysaccharide
A.nodosum	22.5±0.1	6.6±0.1	1.2±0.1	69.6±0.2
F.vesiculosus	24.8±0.2	8.1±0.1	1.4±0.1	65.7±0.4

(Referred source: Rioux, 2005)

1.5.2 Geographical distribution of *F. vesiculosus* and *A. nodosum*

The distribution of *F. vesiculosus* and *A. nodosum* is mainly in the shores of North America (USA and Canada) and North Atlantic Europe countries in North Atlantic Ocean. Cooler temperatures appear to be required for the colonization of shores by these algae. According to National Fisheries Research and Development Institute of Korea, the habitat of these algae is not found on the shore of Asian countries like Korea and Japan. It is rare to find *F. vesiculosus* and *A. nodosum* in other Asian countries

In Canada, *F. vesiculosus* and *A. nodosum* are most abundant on the southern shores of the Saint-Lawrence's gulf in the Province of Quebec (Fig. 1-2). However, the harvesting period is very limited compared to other regions because of the long winter. A thick layer of ice covers the Saint Lawrence River as early as December for almost half year, limiting the seaweed harvest to the late spring and the summer and autumn.

Figure 1-2. The habitat of *F. vesiculosus* and *A. nodosum* in Quebec (Fisheries and Oceans Canada).

1.6 Fucoidan

1.6.1 Characteristics

Fucoidan was first extracted in 1913 from *Laminaria digitata, Fucus vesiculosus* and *Ascophyllum nodosum* (Li et al., 2008; Percival and McDowell, 1967). Fucoidan can only be found in brown algae, not in red or green algae (Percival and McDowell, 1967). Fucoidan is a mucilaginous negatively charged polysaccharide highly hygroscopic (Percival and McDowell, 1967). Higher content of fucoidan is found in surface of leaves of *Laminaria digitata, Ascophyllum nodosum, Macrocystis pyrifera* and *Fucus vesiculosus* (Evans and Callow, 1974). Fucoidan is soluble in water and in acid solution (Rupérez et al. 2002). It is generally believed that the function of fucoidan is to prevent the surface of the seaweed from drying out when the algae are exposed to air above the water surface (Park et al, 1997; Percival and McDowell, 1967).

The exact molecular mass of fucoidan is still debated. The molecular weight (Mw) of fucoidan was different depending on seaweed species and extracting method. Mw of fucoidan from A. *nodosum* was 417 kDa and 1,323 kDa. In the case of *F. vesiculosus*, it was 529 kDa and 887 kDa (Rioux et al., 2007a). Patankar et al. (1993) reported 100 kDa. Rupérez et al. (2002) extracted two fucoidan fractions with different molecular weight (1,600 kDa and 43 kDa). In addition, galactofucoidan, a kind of fucoidan obtained from *Saccharina longicruris* had variable molecular weights between 765 and 1,529kDa, depending on seaweed harvesting time (Rioux et al., 2010). Molecular mass appears to be affected by seaweed species, extraction method and environmental conditions. Fucoidan in the native form can be bound to protein (Tissot et al., 2003).

1.6.2 Composition

The early reports on fucoidan composition defined it as a polysaccharide found in a hydrophilic extract containing L-fucose and D-xylose, while D-galactose and uronic acid were considered contaminants (Percival and McDowell, 1967). However, Percival and Ross (1950) reported 38% half ester sulfate, 56.7% fucose, 4% galactose, 1.5% xylose, 3% uronic acid and 8% mineral in hot water extracts of fucoidan from *Fucus vesiculosus, Fucus spiralis* and *Himanthalia lorea*. Although many components besides fucose and sulfate were detected in this study, it was not confirmed until later by Bernady and Springer (1962) and O'Neill (1954) that fucoidan itself could to be made of more than

just fucose. The fucose to galactose ratio was shown to be dependent on species (Nishino et al., 1994; Marais and Joseleau, 2001; Percival and McDowell, 1967). Dillon et al. (1953) separated fucoidan from *A. nodosum* with a fucose to galactose ratio of 8:1, while a ratio of 18:1 was found for fucoidan extracted from *Macrocystis pyrifera* (Schweiger, 1962). In addition, other types of monosaccharide sugars (e.g. xylose) were recognized as components of fucoidan (Percival and McDowell, 1967). The composition determined for fucoidan from *F. vesiculosus* was 44.1% fucose, 26.3% sulfate, 31.1% ash (Percival and Ross, 1950) and a small amount of amino-glucose (Li et al., 2008). Therefore, the composition of fucoidan can be different depending on species and extraction method (Mian and Percival., 1973; Brasch et al., 1981; Marais and Joseleau., 2001).

1.6.3 Concentration in algae

Opinions still vary on the subject of the seasonal variation of the fucoidan content of brown algae. Park et al. (1997a) reported that fucoidan content was 1~20% of dry seaweed, depending on species. Koo (1994) had earlier reported that *L. religiosa*, *U. pinnatifida*, *H. fusiforme* and *S. fulvellum* contained respectively only 2.7%, 6.7%, 2.5% and 1.6% pure fucoidan. Stewart et al. (1961) reported that fucoidan content did not differ significantly over the seasons, even though the quantity of alginate and laminaran did. However, this finding was later corrected in numerous studies, for example, the fucoidan content of *L. longicruris* in Quebec varied depending on harvesting month (Souchet, 2004). The fucoidan content differs by region, algal species and the season (Park et al., 1997a; Usov et al., 2001; Mian and Percival, 1973).

1.6.4 Structure

Fucoidan is a family of sulfated homo- and hetero-polysaccharides and is composed mainly of α-(1-2)- or α-(1-3)-linked L-fucose residues (Patankar et al., 1993; Percival and McDowell; 1967). A lot of fucoidan's structure analysis has been reported and various structural characteristics depend on algal species. The precise structure of the fucoidans from *Fucus vesiculosus* and *Ascophyllum nodosum* (Berteau and Mulloy, 2003) remains uncertain (Fig. 1-3), although the main repeating unit has been confirmed.

The perceived structure of this fucoidan has changed over the years with the findings of several studies. It was reported first by Conchie & Percival (1950) and O'Neil (1954) that the primary structure of fucoidan was α-(1-2)-linked fucose-4-sulfate with the possibility of α-(1-3)-linked fucose-4-sulfate branches. However, Patankar et al. (1993) corrected this model, as shown in Figure 1-4, suggesting α-(1-3)-linked fucose as a backbone with sulfate groups at C-4 on every two or three fucose residues within the chain (Fig. 1-3). The consensus remains that sulfate is located mostly at C-4 (Patankar et al., 1993) and mostly on fucose residues (Conchie and Percival, 1950; O'Neil, 1954) in fucoidan obtained from *F. vesiculosus*. Anno et al. (1970) reported that the sulfate group was at the axial C-4 position.

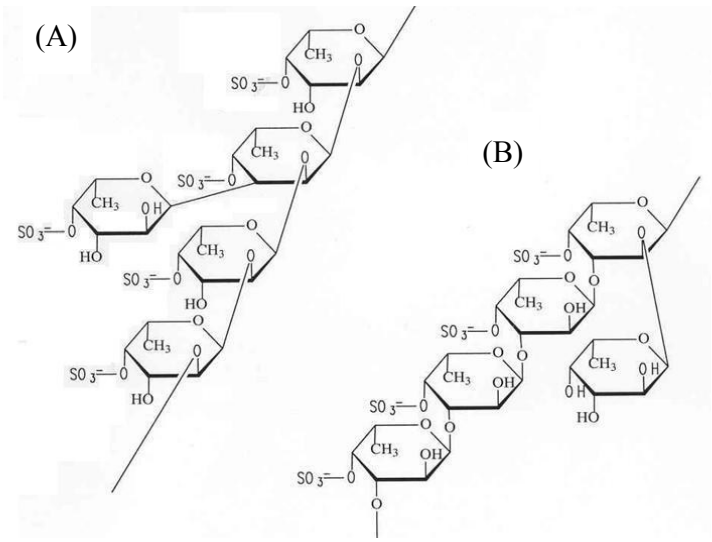

Figure 1-3. The proposed structures of fucoidan structures

(A) Fucoidan structures proposed by Percival and McDowell (1967), (B) Fucoidan structures proposed by Patankar et al. (1993)

As shown in Bilan et al. (2002), the fucoidan obtained from different species of *Fucus evanescens C. Ag, Fucus distichus* and *Fucus serratus L*. contains (1-3)-α-L-Fuc($2SO_3^-$)-(1-4)-α-L-Fuc($2SO_3^-$)- with additional sulfate at C-4 of some 3-linked fucose residues on a linear backbone of 3-linked and 4-linked alpha-L-fucose-2-sulfate residues. The sulfate groups are mostly at C-2 and often at C-4 of

fucoidan from *F. serratus,* while 3,4-diglycosylated and many terminal fucose residues are not sulfated.

The fucoidan extracted from *Stoechospermum marginatum* has (1-4)-linked and (1-3)-linked-α-L-fucosyl residues as a backbone and sulfates at C-2 and/or C-4 of the fucosyl residues (Adhikaria et al., 2006). However, a mixture of α-(1-2)-linked and α-(1-4)-linked fucose is also found in some brown algae (Li et al., 2008). Mian and Percival (1973) reported that the fucoidan from *Himanthalia lorea* and *Bifurcaria bifurcate* was (1-2)-linked or (1-3)-linked, while that from *Padina pavonia* contained (1-2) and (1-4) linkage.

The fucoidan extracted from *Ascophyllum nodosum* is composed of [1-3)-α-L-Fuc($2SO_3^-$)-(1-4)-α-L-Fuc(2,3diSo_3^-)-(1]*n* (Chevolot et al., 2001). The main structure is a α-(1-2)-fucose backbone with a high proportion of α-(1-4)-linkages, while branch points are from the second position of -3-fucosyl internal residues. Sulfate groups occur at C-2 and/or C-4 (Chevolot et al., 1999; Chevolot et al., 2001; Marais and Joseleau, 2001). Moreover, as shown in Figure 1-5, α-(1-3) and α-(1-4) glycosidic bonds were detected in oligosaccharides of 8-14 monosaccharide units in fucoidan from *A. nodosum* (Chevolot et al., 2001) and a highly branched fucoidan fraction was also obtained from this species (Marais and Joseleau, 2001). In addition, (1-3)-linked and (1-4)-linked un-sulfated and 2-sulfated α-L-fucose residues were found in low molecular mass fragments of fucoidan obtained by enzymatic degradation (Daniel et al. 1999).

In summary, fucoidan structure is difficult to define with precision because of the variability of the linkage types among the different algal species. However, the position of sulfate group on fucose has been confirmed at least for *F. vesiculosus* and *A. nodosum*.

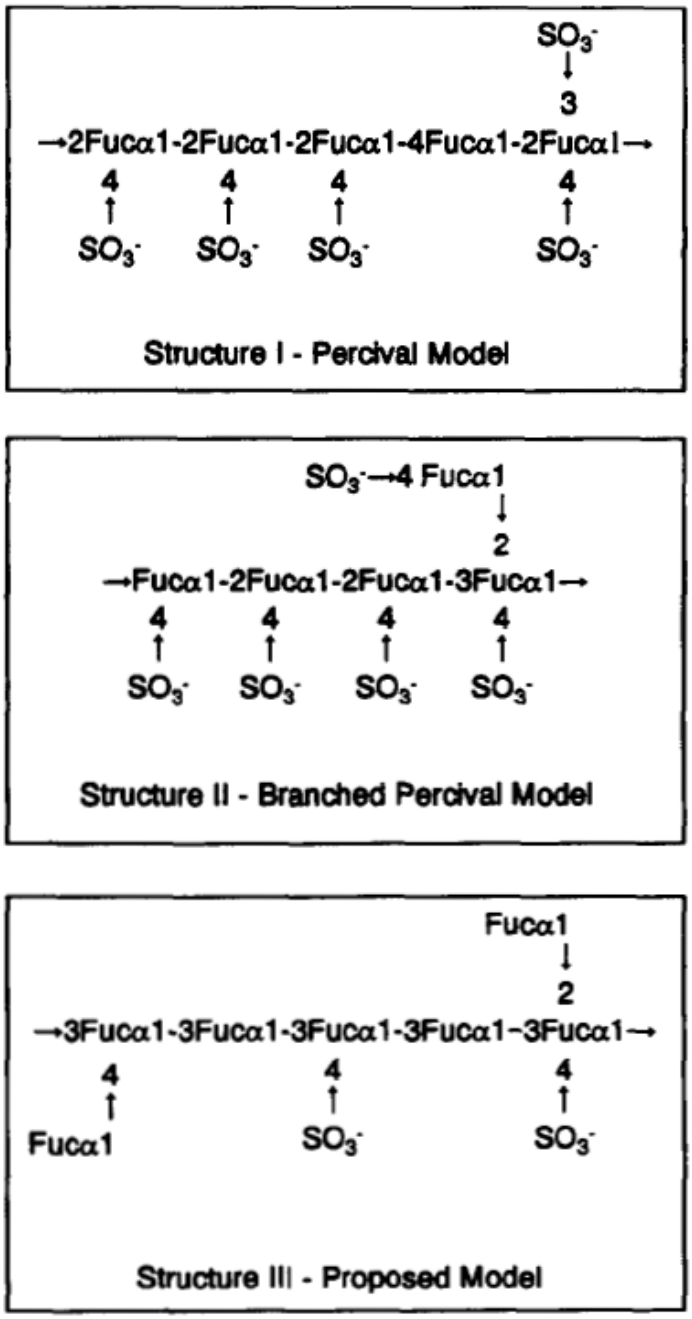

Figure 1-4. Models for the overall structure of fucoidan from *F. vesiculosus*
(Reference; Patankar et al., 1993)

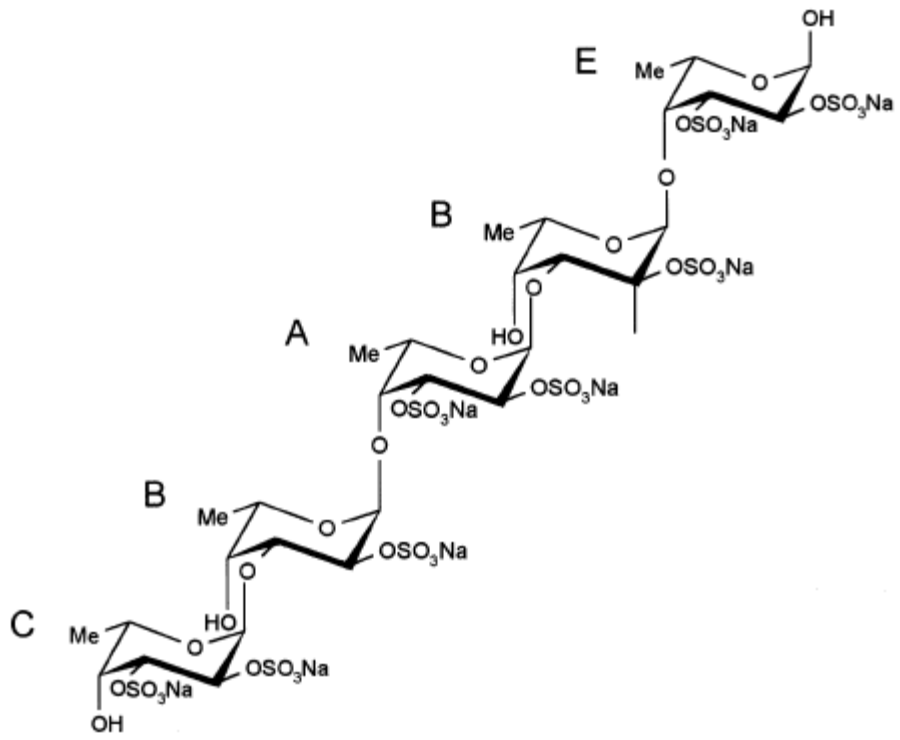

Figure 1-5. Structure of fucoidan from *A. nodosum*

(Reference; Chevolot et al., 2001)

1.6.5 Sulfate groups and their relationship with biological activities

In general, the sulfate content of fucoidan from brown algae including *F. vesiculosus* and *A. nodosum*, is 35-40% of the dry weight of the fucoidan (Kloareg et al., 1986). Fucoidan is always described as a 'sulfated polysaccharide' or 'polysaccharide containing sulfate groups'. Why so much emphasis on sulfate groups? Sulfate is one of the main structural or chemical factors associated with biologically or physiologically active polysaccharides. For example, heparin is a clinical anticoagulant (Murray, 1947) and its sulfate content is associated with its anticoagulant activity (Stringer and Gallagher, 1997). Soeda et al. (2000) demonstrated the increased ability of over-sulfated fucoidan to bind to and inhibit bFGF (basic fibroblast growth factor). The number of sulfate groups in fucoidan could contribute to the efficacy of its anti-angiogenic and anti-tumor activities (Koyanagi et al., 2003). Furthermore, low molecular weight fucoidan (fucan) from *A. nodosum* is involved in anticoagulant and anti-proliferative activities and these properties depended also on the number of sulfate groups (Haroun-Bouhedja et al., 2000; Roger et al., 2004). In another case, the sulfonated residues of laminarin were effective in preventing and treating ischemic cerebrovascular disease (Miao et al., 1995). Dextran sulfate has displayed some ability to inhibit HIV (human immunodeficiency virus) (Flexner et al., 1991).

In general, infrared (IR) analysis (Qiu et al., 2006) and inductively coupled plasma-optical emission spectroscopy (ICP-OES) (Roger et al., 2004) can be used to measure sulfate group content. Total sulfate measurement can be done using ICP-OES with the equation shown in Rioux et al. (2007), while the position and type of sulfate group can be determined by IR analysis (Mähner et al., 2001; Yang et al., 2003; Lijour et al., 1994; Rupérez et al., 2002). In the case of fucoidan, the asymmetrical stretching of S=O bonds resonate at 1250 cm^{-1} and CSO at C-2 or C-3 resonate at 820 and C-4 at 840 cm^{-1} (Qiu et al., 2006; Patankar et al., 1993).

1.6.6 Biological activity of fucoidan

1.6.6.1 Anticoagulant (anti-thrombin) activity

Springer et al. (1957), who extracted fucoidan from *Fucus vesiculosus*, discovered the blood anticoagulant activity of this polysaccharide. This activity depends on the molecular mass and the number of sulfate groups. In general, the maximum anti-thrombin activity is observed with fucoidan

of mass between 10 and 30 kDa (Zvyagintseva et al., 1999). When the number of sulfate groups increases, the activity increases (Li et al., 2008). Fucoidan inhibits the conversion of prothrombin to thrombin and this is caused by α(1→2)-L-fucose-sulfate residues of the third and fourth carbon (Pereira et al., 1999). The reaction of fucoidan is similar to the blood anticoagulant heparin. Heparin is hydrophilic and activates lipase in order to hasten the circulation of lipids in blood vessels (Soeda et al., 2000; Nishino and Nagumo, 1992).

1.6.6.2 Anti-HIV activity

Fucoidan has been shown to have some anti-HIV activity. The human immunodeficiency virus (HIV) is a cause of acquired immune deficiency syndrome (AIDS). The proposed mechanism of anti-HIV activity is that fucoidan acts as competitive inhibitor of the interaction between HIV reverse transcriptase and the nucleic acid substrate (Moen and Clark, 1993; Schaeffer and Krylov, 2000). However, the anti-HIV activity of fucoidan is still debated because it has been also reported that fucoidan does not inhibit HIV syncytium formation (Béress et al., 1993; Moen and Clark, 1993; Schaeffer and Krylov, 2000).

1.6.6.3 Angiogenesis activity

Angiogenesis is divided into pro- and anti-angiogenesis. Pro-angiogenesis activity promotes the progression of new blood vessels. Low-molecular-weight fucoidan especially has shown pro-angiogenesis activity in endothelial progenitor cell (EPC) enhancement (Zemani et al., 2005). Anti-angiogenesis is interference with or inhibition of blood vessel cell multiplication and is related to anti-cancer activity. Furthermore, the direct interaction between fucoidan and protein (as a major content of blood vessel cells) has been demonstrated by affinity capillary electrophoresis.

1.6.6.4 Anti-tumor (anti-cancer) activity

Anti-tumor activity has been reported in numerous studies (Koyanagi et al. 2003; Cho et al. 2011b; Synytsya et al. 2010; Yang et al., 2008). Both normal fucoidan and over-sulfated fucoidan suppress the activity of vascular endothelial growth factor 165($VEGF_{165}$) by inhibiting the binding between

$VEGF_{165}$ and its cell surface receptor (Koyanagi et al., 2003). In addition, this inhibitory activity increases in proportion to the number of sulfate groups in the fucoidan structure (Koyanagi et al, 2003; Zvyagintseva et al,).

1.6.6.5. α-Amylase inhibitory activity

Cho et al. (2011a) recently studied the inhibitive activity of fucoidan extracted from *Undaria pinnatifida*. The authors showed that native fucoidan (containg 41.5% of sulfate) had no effect on α-amylase activity. However chemically oversulfated fucoidan (51.1% of sulfation) decreased amylase activity. Furthermore, both native and oversulfated fucoidans reduced α-amyloglucosidase activity.

1.6.7 Toxicity of fucoidan

Even positive functional material and compound might cause a side-effect and toxicity when it would be over-dosed or inappropriately used. For example, dextran sulfate, a polysaccharide having a large molecular mass, inhibits a sort of reverse transcriptase (RT). It is also toxic when injected intravenously and reduces reversible alopecia (Flexner et al., 1991). Li et al. (2005) reported that fucoidan was not toxic below 300 mg/kg/day, but can be toxic at higher intake of 900 ~ 2,500 mg/kg/day. Therefore, it may be necessary to consider the range of toxicity of fucoidans when designing safe applications for them.

Among the biological activities of fucoidan, inhibition of amylases is very interesting because it is closely related to diabetes. Recently starch digestive enzymes inhibitors (including amylase and glucosidase) like acarbose have been studied to control diabetic symptom by delay or interruption of glucose absorption.

1.7 Diabetes

1.7.1 Glucose metabolism

Glucose as a monosaccharide is an important energy source of metabolism and only this form can be used in cells. Several plants store glucose for future utilization as starch. The use of starch as energy source, involves an enzymatic hydrolysis to release free glucose. For starch digestion in the human body, two enzymes are involved, which are α-amylase and α-glucosidase. At first, starch is degraded into large number of maltose by salivary α-amylase in mouth and then the maltose is decomposed into two glucose units in the intestine by α-glucosidase.

Glucose is absorbed from the small intestine into the bloodstream. From the bloodstream, glucose is transported into the liver and muscles through a process mediated by insulin, which is a hormone secreted by the pancreas. The normal level of blood glucose is about 80 mg/dl between meals and up to 110 mg/dl shortly after a meal. Even when large quanties of starch are consumed, the level of blood glucose does not exceed 140 mg/dl in normal humans. In diabetic patients, however, blood glucose may exceed 140 mg/dl even between meals due to an impaired capacity to regulate glucose level (Porte, 2001).

The WHO report in 1999 upheld the classification of diabetes in two categories: type 1 and type 2. Type-1 diabetes is due to little or no insulin secretion from the pancreas and is thus named insulin-dependent diabetes mellitus. Type-1 diabetes usually involves hereditary factors. Type-2 diabetes is non-insulin dependent and is the result of insufficient insulin secretion for the amount of blood glucose absorbed. Most diabetic patients have type-2 diabetes and over 80% of these persons are obese (Kuo et al., 2008). Type 2 is considered a more serious problem for society as a whole, since it is increasing in prevalence very quickly and threatens to become a huge burden on health-care systems. According to the study by Wild et al. (2004), the number of diabetic patients in the world was 171 million in 2000 and will be 366 million in 2030. It is assumed that the increase in cases will be about 6.5 million per year if nothing is done to stop it.

1.7.2 Symptoms and complications of diabetes

Diagnosis of type-1 and type-2 diabetes is based on the level of glucose in blood. Excess blood glucose increases blood viscosity, decreases blood flow speed and increases blood pressure (Clinical

Pratice Guidelines of Canadian Diabetes association; http://www.diabetes.ca/diabetes-and-you/what/facts/). Glucose has four hydroxyl groups available for hydrogen bonding and its reducing end is a very reactive chemical group, providing numerous possibilities for accelerating the precipitation of other blood components such as lipids and nutrients. At the extreme, sludge will block blood vessels and the circulation of blood, oxygen and other nutrients into cells (Grant, 2010). Diabetes causes a complex disease, a metabolic problem and serious damage to cells and the nervous system. Blockage of blood vessels is frequent and is more serious in the fingers and toes because of the number of capillary vessels. Also, diabetes predispose to several diseases like cardiovascular, necrosis, Alzheimer (Marks and Raskin, 2000; Kuo et al., 2008; Kloppenborg et al., 2008; Biessels and Kappelle, 2005).

1.7.3 Cure and treatment of diabetes: limitation of medications and diet

The development of medication for diabetes has focused mostly on type-2 diabetes, because insulin injection remains the only way possible to treat type-1 diabetes. A clinical approach is possible and preferred for ameliorating the symptoms of type-2 diabetes. In order to cure diabetes, especially type 2, there are currently two main approaches, namely medication and diet therapy. Insulin injection has provided a simple and effective medical cure. However, this is expensive and uncomfortable. In addition, insulin efficiency falls to 50% within three hours after injection (Canadian Diabetes Association, 2008). Moreover, frequent utilization of insulin can cause insulin resistance and overload of the liver (Porte, 2001; Kahn and Porte, 1997; Reaven, 1988). Stimulation of insulin secretion therefore has been studied as an alternative to insulin injection and effective compounds such as sulfonylurea and repaglinide have been developed (Daniel, 2001). However, these stimulants are not free of side effects and their effectiveness is short-lived (Daniel, 2001).

Control of nutrient intake is the method most recommended by physicians and pharmacists for curing diabetics. This can stabilize the level of blood glucose even without medication. This is called diet or medical nutrition therapy (MNT) and is implemented using the glycemic (blood sugar raising) index or GI of foods. Low GI foods have been shown to decrease the postprandial blood glucose peak, to increase satiety, to promote weight loss, to improve insulin sensitivity and to improve lipid profile (Jenkins et al., 2002; Leeds, 2002; Brand-Miller et al., 2002). The MNT diet approach is effective, simple and helpful for curing diabetes and maximizing the effectiveness of medication (Choudhary,

2004). However, MNT/diet is not an ideal solution for diabetics because it places serious limitations on the quantity and variety of foods consumed in order to comply with the GI constraint. Limiting food consumption deprives the patient of the major pleasures of enjoying food taste and satisfying appetite. This limitation can also affect friends and family of the diabetic patient from time to time. It can induce stress by suppression of natural desire. Diabetic patients thus cure their condition by sacrificing their quality of life.

To avoid such negative effects such as these, many food scientists and medical researchers have looked for alternatives to improve the quality of life and minimize the drawbacks of MNT, namely delaying or interrupting glucose absorption. This approach could make more flexible food consumption possible and thereby improve the quality of the diabetic patient's life. For this purpose, the focus has been on inhibiting starch digestive enzymes.

1.8 Starch digestive enzymes

1.8.1 Salivary α-amylase

Amylase is defined as an enzyme that hydrolyzes the O-glycosyl linkages of starch. There are four categories of amylase, based on the type of linkage hydrolyzed and the type of molecule released or synthesized: (1) α-amylase, which hydrolyses α-1,4-glucosidic linkages (EC 3.2.1.1); (2) pullulanase (EC 3.2.1.41) or isoamylase (EC 3.2.1.68), which hydrolyse α-1,6-glucosidic linkages; (3) cyclodextrin glucanotransferase or CGTase (EC 2.4.1.19), which catalyzes transglycosylation to form α-1,4-glucosidic linkages and (4) 1,4-α-D-glycan/6-α-D-(1,4-α-D-glucano) transferase or branching enzyme (EC 2.4.1.18), which catalyzes transglycosylation to branches (Kuriki and Imanaka, 1999).

Alpha-amylase is one of the major protein components of saliva. The α-amylase in human saliva (E.C. 3.2.1.1.) is an example of a category-1 amylase (Hara and Honda, 1990). This enzyme catalyzes random splitting of α-1,4 glucosidic linkages of glucans and the terminal of glucose bond is split much more slowly (Bernfeld, 1955). The end products of amylolytic digestion of starches are maltose and some glucose (Jacobsen et al., 1972; Baum, 1993). The hydrolysis of starch by α-amylase thus starts during mastication. Gastric acid stops this activity.

The molecular mass of salivary α-amylase is approximately 55 kDa. The optimal pH for its hydrolytic activity is 6.8 and its isoelectric point is about pH 8.0 (Righetti and Caravaggio, 1976; Jacobsen et al., 1972). The concentration of amylase in human saliva is generally in the 0.04-0.4 mg/ml range and this varies slightly with the overall condition of the individual, age and period of the day and with food consumption (Jacobsen et al., 1972)

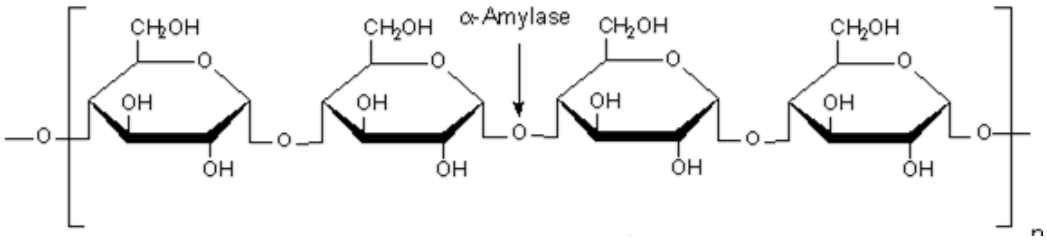

Figure 1-6. Starch hydrolysis by α-amylase

Although α-amylase is a very well known enzyme, some details about its function remain unclear (Dolečková-Marešová et al., 2005). Based on existing knowledge, α-amylase is activated by divalent cations, especially calcium (Ca^{2+}), which is an important stabilizing factor in many proteins and a well-known α-amylase stabilizer. The α-amylase molecule comprises three domains, A, B and C. Domain A is the largest and contains the active site near the carboxyl-terminal end of the β-strands. Once believed to bind strongly between domains A and B, calcium has been shown to bind to a high-affinity site and to a secondary binding site near the active site (Teeri, 1991).

Like Ca^{2+}, chloride ion (Cl^-) also contributes to α-amylase stabilization, and can even increase α-amylase activity (Jacobsen et al., 1972; Bellavia et al., 1979). As little as 1 mM Cl^- restored up to 80% of the initial activity of α-amylase lost caused by purification steps (Bernfeld, 1955).

1.8.1.1 Inhibitors of α-amylase

1.8.1.1.1 The purpose of α-amylase inhibition

The inhibition of α-amylase contributes to improve symptoms of type-2 diabetes (Lamela et al., 1989) by delaying or interrupting glucose absorption as a result of slowing starch digestion. Although

the main purpose of α-amylase inhibition is to slow down maltose and glucose production, it can also slow α-glucosidase function by eliminating the substrate of this enzyme. Golay et al. (1991) demonstrated the improvement of diabetes mellitus by the delay of starch disgestion by adding trestatin which is partially purified mixture of complex oligosaccharides and α-amylase inhibitor.

1.8.1.1.2 Mechanisms of amylase inhibition

The mechanism of α-amylase inhibition differs somewhat depending on inhibitor type. Suggested inhibition mechanisms are (1) an inhibitor combines with the negatively charged binding/catalytic site of α-amylase (Kim et al., 1999; Hansawasdi et al., 2000; Nahoum et al., 2000; Qian et al., 2001) as is the case for inhibitors such as acarbose, isoacarbose, acarviosine-glucose, hibiscus acid and cyclodextrins (Fig.1-7); (2) blocking the network of hydrogen bonding between the substrate and the amino acid residues of the substrate-binding region; (3) bifunctional inhibition in which the inhibitor fits between α-amylase and the substrate (Alam et al., 2001; Strobl et al., 1995); (4) slowing the diffusion of glucose from the active site, for example, by viscous water-soluble dietary fibers, delaying both carbohydrate digestion and glucose absorption (Ou et al., 2001); (5) binding to another site (site 1 or 2 in Fig. 1-8). In this latter case, α-amylase does not function even though it binds with its substrate (Ferey-Roux et al., 1998). (6) Complex formation with large molecular weight macromolecule as polyphenol and polysaccharide. Several natural compounds have inhibitory effect on α-amylase. They are presented below.

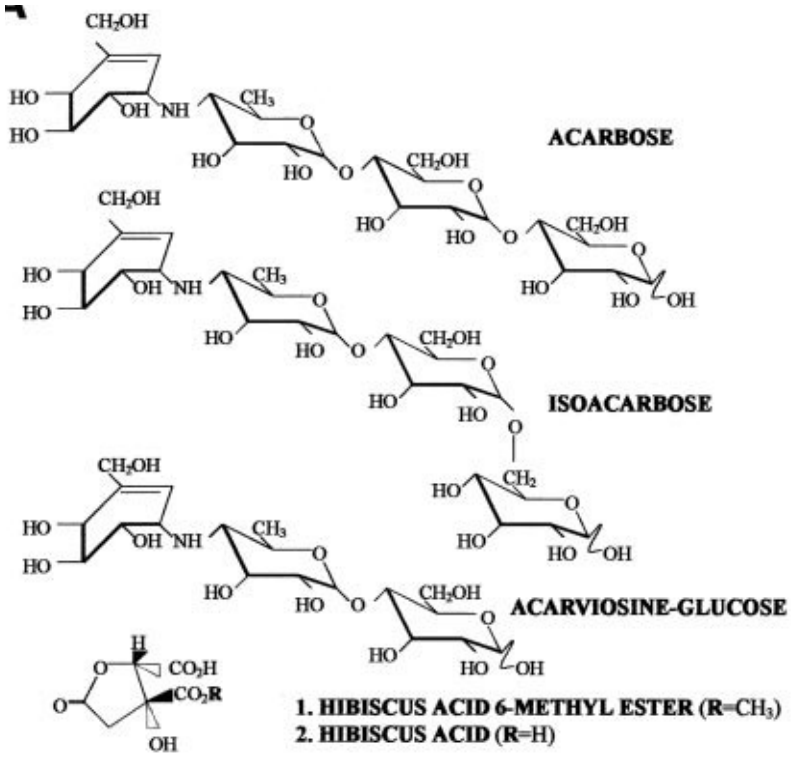

Figure 1-7. Structure of the α-amylase inhibitors acarbose, isoacarbose and acarviosine-glucose

(Reference; Franco et al., 2002)

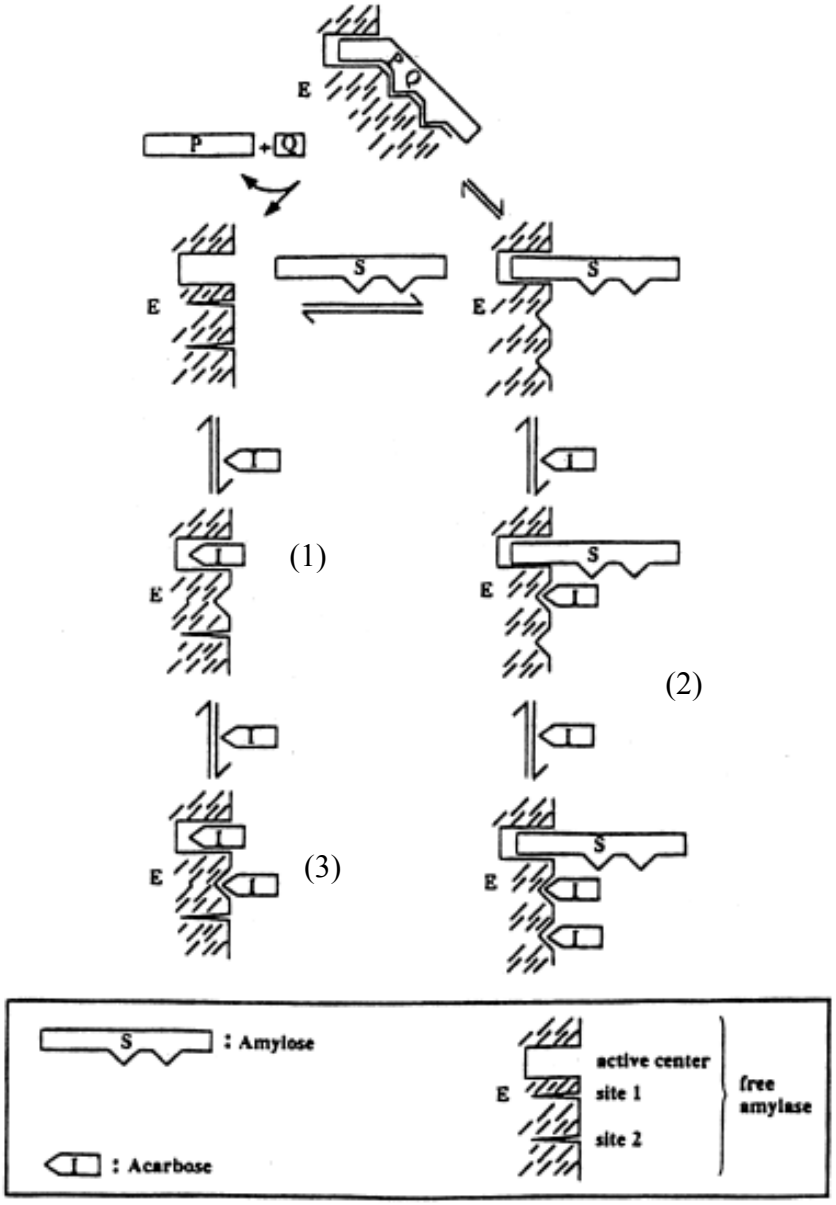

Figure 1-8. Mechanism of α-amylase inhibition by acarbose

(Reference; Ferey-Roux et al., 1998)

In the inhibition of α-amylase by acarbose, 3 mechanisms are possible: acarbose can bind to (1) only the active site of α-amylase for substrate, (2) other active sites of α-amylase, not for substrate, and (3) active sites of (1) and (2) at the same time.

1.8.1.1.3 Polyphenols

Polyphenols including tea polyphenols are α-amylase inhibitors in addition to being strong antioxidants. However, the exact mechanism of α-amylase inhibition by polyphenols is not yet fully understood. The mechanism of α-amylase inhibition could be as suggested by Spencer et al. (1988), who reported that phenolic polymers combine with protein and thus inhibit enzymes as well as reducing the nutritional availability of proteins in foods. The association of polyphenols with proteins is principally a surface phenomenon, as shown in Figure 1-9. The efficacy of polyphenol as inhibitors is related to its ability to bind simultaneously to more than one point on the protein surface. By binding in monolayers to protein surface, polyphenols reduce the hydrophilic character of proteins and cause their precipitation even at low concentrations (Spencer et al., 1988) (Fig. 1-9a). At high protein concentrations, polyphenols use their multi-dentate [1] nature to form a cross-linked hydrophobic surface layer with protein molecules and thereby cause precipitation (Spencer et al., 1988) (Fig. 1-9b).

In addition, He et al. (2006a and 2006b) suggested that α-amylase inhibition by tea polyphenols occurs as a result of hydrogen bonding with the polar groups (amide, guanidine, peptide, amino and carboxyl groups) of the enzyme. Then, the mechanisms of α-amylase inhibition are diverse and depend on various factors as enzyme concentration, specificity of the binding site, ligand nature and so on.

1.8.1.1.3.1 Tannins

Tannins are natural products with relatively high molecular weight which have the ability to bind strongly to carbohydrates and proteins including α-amylase (Park et al., 1997a; Park et al., 2000; Al-Mamary et al., 2001). Tannins belong to a broad class of chemicals called polyphenols and are widespread in plant-based foods such as fruits, vegetables, teas and beverages. Tannins can be divided into two groups, hydrolyzable and condensed tannins (Park et al., 1997a; Park et al., 2000). Hydrolyzable tannin is subdivided into gallotannin and ellagitannin based on the nature of the phenolic carboxylic acid (Park et al., 1997a; Park et al., 2000). Kandra et al. (2004) demonstrated that gallotannin (containing quinic acid with two to seven units of gallic acid) inhibited 2-chloro-4-nitrophenyl-4-O-β-D-galactopyranosyl-maltoside hydrolysis catalyzed by human salivary α-amylase,

[1] being, containing, or involving a ligand that can form bonds at more than one point

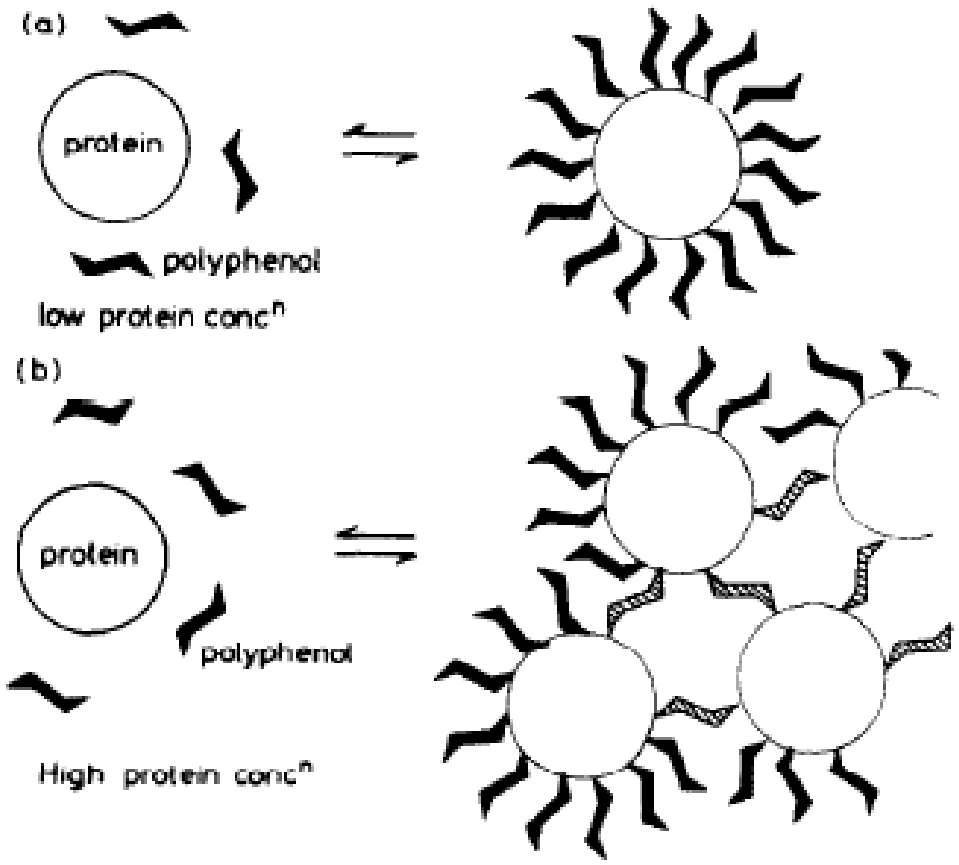

Figure 1-9. Polyphenol-protein complexation and precipitation
(Reference; Spencer et al., 1988)

with kinetic constants K_{EI} = 9.03 μg/mL (complex of enzyme and inhibitor) and K_{ESI} = 47.8 μg/mL (complex of enzyme, substance and inhibitor). Low value of kinetic contant (K) indicates high affinity with enzyme. In addition, tannins are known to be potential metal ion chelators, protein precipitating agents and biological antioxidants.

1.8.1.1.3.2 Tea polyphenols

Tea polyphenols are believed to have biological properties such as decreasing the risk of heart disease and cancer and acting as antioxidants (Park et al., 1997a; Park et al., 2000). Based on their ability to bind to proteins, tea polyphenols could interfere with the activity of digestive enzymes. In the presence of 0.05 mg/mL of polyphenols extracted from Chinese green tea, the activities of α-amylase, pepsin, trypsin and lipase were decreased respectively by 61%, 32%, 38% and 54% (He et

al., 2006a). According to He et al. (2006a and 2006b), tea polyphenols can reduce α-amylase activity by 39%.

1.8.1.1.3.3 Polyphenols in brown algae

Brown seaweeds such as *A. nodosum, F. vesiculosus, F. serratus* and *Pelvetia canaliculata* are reported as good sources of polyphenols. Polyphenols extracted from brown seaweeds at a concentration of 0.05mg/ml decreased α-amylase activity by 90%. The α-amylase inhibiting potency was twice as high per gram of *F. vesiculosus* as for *A. nodosum, F. serratus* and *P. canadiculata* (Barwell et al., 1989). The potential function of polyphenols of *A. nodosum* as starch digestive enzyme inhibitor was also reported by Zhang et al. (2007) and Apostolidis and Lee (2010).

1.8.1.1.4 Fucoidan

Recently, Cho et al. (2011a) reported that chemically oversulfated fucoidan from *Undaria pinnatifida* inhibited α-amylase however native fucoidan did not. The authors proposed that the inhibition ability of oversulfated fucoidan was due to better mobility and higher diffusion associated to lower viscosity of oversulfated fucoidan. Interestingly, the difference of sulfate content between oversulfated and native fucoidan was only 10%.

1.8.2 α-Glucosidase

Alpha-glucosidase (EC. 3.2.1.20, α-D-glucosidic glucohydrolase) is the other main starch-digesting enzyme and is considered a target for the amelioration of type-2 diabetes. Many studies on inhibiting this enzyme have been done. Alpha-glucosidase is an exo-type carbohydrase that liberates glucose by catalyzing the hydrolysis of the α-(1,4)-glucosidic linkage at the non-reducing end of the substrate (Yu et al., 1999). In plants, α-glucosidase is a defense mechanism that works by releasing inhibitory compounds that are stored in vacuoles as stable, highly soluble and non-active glucosylated forms (Jones and Vogt, 2001). Alpha-glucosidase thus catalyzes the final step of starch digestion, its substrates being the products of the action of α-amylase (Fig. 1-10). In type-2 diabetes, inhibition of α-glucosidase to delay the absorption of glucose is well known to be beneficial in therapy. This

enzyme is therefore a major target in the design and development of anti-diabetic therapy (Tewari et al., 2003).

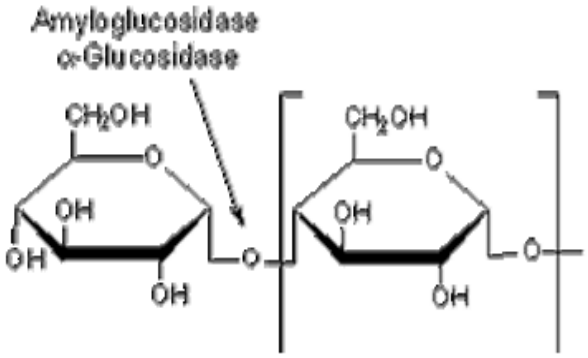

Figure 1-10. Catalysis of maltose hydrolysis by α-glucosidase
(Reference; Sigma-Aldrich, 2007)

1.8.2.1 Mechanism of α-glucosidase action

Most glucosidases contain a pair of carboxylic acid groups (-COOH) in the active site. Two theories have been advanced to explain the glucosidase activation mechanism: one is an inverting reaction mechanism and the other is a retaining mechanism. The two mechanisms differ by the replacement process being direct or indirect. In the inverting reaction, a part of the enzyme acts as an acid and the other is generally a base. The reaction occurs by direct displacement, the position of carboxyl group hydrogen (H) of glucosidase is transferred to the other side of the enzyme after the hydrolysis (Fig. 1-11, A). In contrast, the retaining reaction involves a double-displacement: a glucosyl-enzyme intermediate involving one of the carboxyl groups is formed and then hydrolyzed with general acid/base assistance from the other group. There is no difference between glucosidase structure at the beginning and the end of the reaction (Fig. 1-11, B). The normal mechanism of α-glucosidase involves the formation and hydrolysis of a glycosyl-enzyme intermediate with general acid/base catalytic assistance via transition states with substantial oxocarbenium ion character (Lai et al., 1996; Sinnott, 1990).

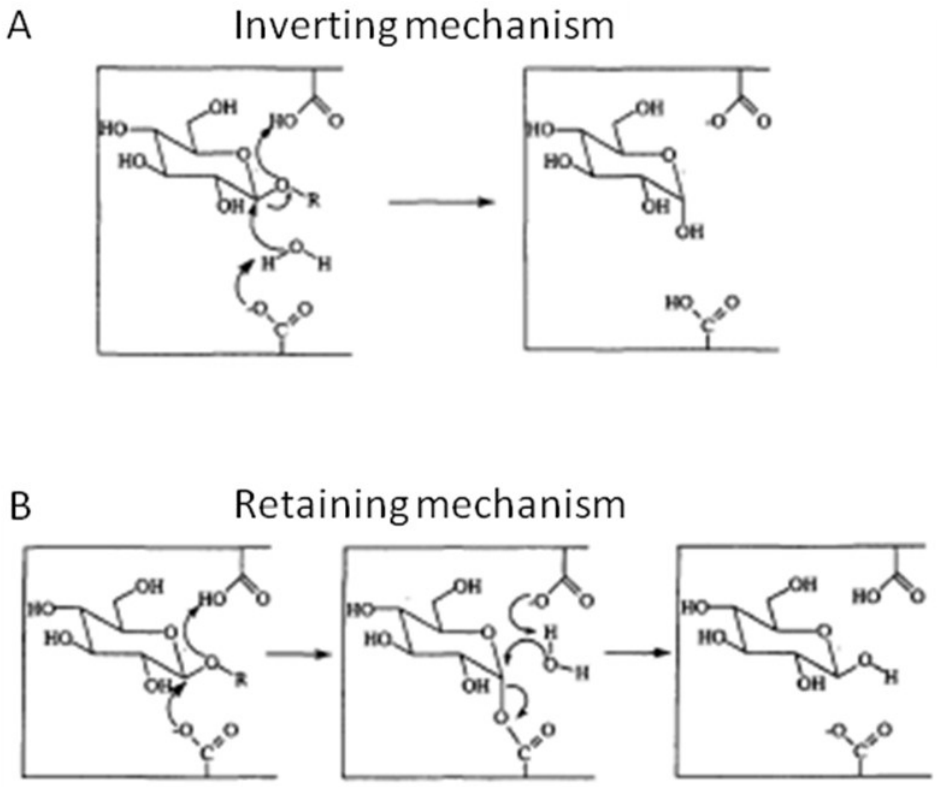

Figure 1-11. Inverting and retaining mechanisms of glucosidase activity

(A) Direct displacement: acid and base sites exist at the same time; part of the enzyme reacts with intermediate (H_2O). (B) Double-displacement: glycosyl-enzyme intermediate involves one of the carboxyl groups; enzyme reacts with the substrate and then gets a hydrogen (H) from the intermediate (H_2O). **(**Reference; Lai et al., 1996)

1.8.2.2 Inhibitors of α-glucosidase

The mechanism of α-glucosidase inhibition is simpler than for α-amylase. In order to catalyze the hydrolysis of the α-(1,4)-glucosidic linkage, glucosidase donates the hydrogen of its carboxyl group to a substrate such as maltose. The main principle of α-glucosidase inhibition is the blocking of the hydrogen displacement with C-2 of the substrate through hydrogen interception. The way the hydrogen displacement is blocked depends on the specific inhibitor. The classification of inhibitors varies among authors and researchers and has not been formalized because the known inhibitors and their derivatives are too varied. For example, the standard of classification of Melo et al. (2006) recognizes 153 sugar types and five categories of α-glucosidase inhibitors and the method of

measuring inhibitory activity is not equal. The extracts from tea or green tea are not purified (Matsui et al., 1996) nor are the polyphenols from seaweeds (Zhang et al., 2007; Lamela et al., 1989; Kim et al., 2008). Inhibitors of α-glucosidase have been extracted from various natural sources such as plants, seeds, seaweeds and tea (Thalapaneni et al., 2008; Shinde et al., 2008; Matsui et al., 1996; Kao et al., 2006) or synthesized (Braunt et al., 1995) like acarbose and salacinol and related analogues (Mohan and Pinto, 2007).

Sugar mimics have also been found to inhibit α-glucosidase activity (Fig. 1-12). Some of these contain sulfate groups (Seo et al., 2005; Minami et al., 2008). In addition, polyphenols (e.g. quercetin, catechins, etc.) also show α-glucosidase inhibitory activity (Seo et al., 2005; Ohta et al., 2002; Lee et al., 2008; Gamberucci et al., 2006). It is believed that the chelating, free radical scavenging and antioxidant abilities of polyphenols (Han et al., 2007) contribute to α-glucosidase inhibition, given the enzymatic mechanism. Some polyphenols such as luteolin and flavonoids have inhibitory activity against both α-amylase and α-glucosidase (Kim et al., 2000; Bhandari et al., 2008).

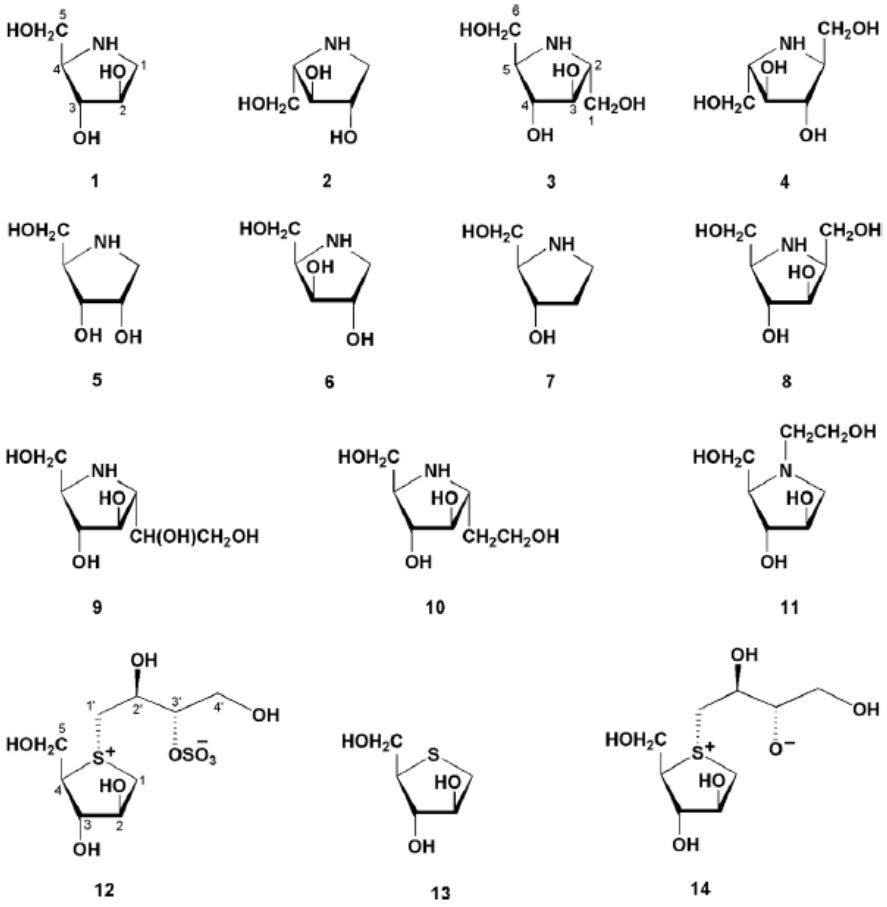

Figure 1-12. Structures of five-membered sugar mimics

1: 1,4-dideoxy-1,4-imino-D-arabinitol (D-AB1); 2: L-AB1; 3: 2,5-dideoxy-2,5-imino-D-mannitol (D-DMD); 4: L-DMD; 5: 1,4-dideoxy-1,4-imino-D-ribitol (D-DRB); 6: 1,4-dideoxy-1,4-imino-D-xylitol (D-DIX); 7: 1,4-imino-1,2,4-trideoxy-D-arabinitol (CYB-1); 8: 2,5-dideoxy-2,5-imino-D-glucitol (D-DIG); 9: 2,5-dideoxy-2,5-imino-D-glycero-D-manno-heptitol (homoDMDP); 10: 2,5-imino-2,5,6-trideoxy-D-manno-heptitol (deoxy-homoDMDP); 11: 1,4-dideoxy-1,4-(hydroxyethyliminiumyl)-D-arabinitol (N-hydroxyethyl-D-DB1); 12: 1,4-dideoxy-1,4-{(S)-[(2S,3S)-2,4-dihydroxy-3-(sulfooxy)butyl]-episulfoniumylidene}-D-arabinitol inner salt (salacinol); 13: 1,4-anhydro-4-thio-D-arabinitol (D-ATA); 14: 1,4-dideoxy-1,4-{(S)-[(2S,3S)-2,4-dihydroxy-3-butyl]episulfoniumylidene}-D-arabinitol inner salt (neosalacinol).

1.9 Hypothesis and objectives

The characteristics and chemical components contents of algae are different depending on seaweed species and region. Algae are a good source of protein and minerals as food and plant fertilizer. Also, seaweed has several molecules such as polyphenols to prevent diabetes by inhibition of starch digestive enzymes activity (α-amylase and α-glucosidase). Study on *F. vesiculosus* and *A. nodosum* brown seaweed growing in the Eastern part of Canada has been few.

The hypothesis of this study is that the understanding of seasonal variation of nutritional components and starch digestive enzymes inhibitory ability of fucoidan will permit to improve the value of seaweed in Quebec (*F. vesiculosus* and *A. nodosum*) and to develop a functional food to control blood glucose.

In order to validate this hypothesis, this study has been performed by three specific objectives;

1. To analyze fucoidan content and nutritional components (protein, lipid, ash, polysaccharide and total phenolic substances) of two brown algae for three different harvest years (2002, 2003 and 2005) from spring to autumn, in order to verify the basic information on seasonal variation of fucoidan and nutritional components depending on harvesting time and species of seaweed.

2. To evaluate the variations of the ability of fucoidan to inhibit starch digestive enzymes (α-amylase and α-glucosidase) depending on harvesting season and seaweed species.

3. To investigate differences between the fucoidans extracted from *Fucus vesiculosus* and *Ascophyllum nodosum* in respect to the inhibition of starch digestive enzymes (α-amylase and α-glucosidase), and to identify major structural factor contributing to the enzyme activity. The obtained information (structure, monosaccharide composition, sulfation and molecular weight) on the variation of fucoidan activity would be helpful for optimization of the use of seaweeds harvested in Quebec; for instance, therapeutic purposes (especially, treatment of type-2 diabetes), functional foods, fucoidan-containing derivatives and so on.

Chapter 2. Seasonal variation of fucoidan and the evaluation of nutritional components of brown algae in Quebec: *Fucus vesiculosus* and *Ascophyllum nodosum*

Kyung-Tae Kim and Sylvie L. Turgeon

2.1 Abstract

F. vesiculosus and *A. nodosum* are eatable brown seaweed and abundant in the province of Quebec. However, the commercial utilization of *F. vesiculosus* and *A. nodosum* is limited. The limited harvesting period, which is caused by a long cold winter, is an obstacle to utilize the seaweeds in Quebec. On the other hand, brown seaweeds have been known to contain numerous functional substances in general. The aim of this study is to investigate the influence of harvesting season for fucoidan and nutritional components of *F. vesiculosus* and *A. nodosum*.

The amount of polysaccharide, protein, minerals, lipid, polyphenols and fucoidan was measured and converted to the relative portion (%, w/w) on dry basis of seaweed. The relative portion of major seaweeds components was ordered as polysaccharide > minerals > protein > lipid > phenol. In a view of nutrition, average content of protein and mineral was similar between *F. vesiculosus* (10.45% ± 1.49% and 25.30 ± 6.30%) and *A. nodosum* (8.41 ± 1.31% and 20.06 ± 3.73%). However, seaweed components showed variable changes between seaweed species depending on season (harvest month). According to seaweed harvesting month, *F. vesiculosus* contained relatively more protein and mineral content than *A. nodosum* and the best harvesting period was in May for protein and summer for mineral respectively. *A. nodosum* had more polysaccharide and fucoidan than *F. vesiculosus* and the optimal period for fucoidan extraction was in July. Interestingly, annual average content of fucoidan was not different among 2002, 2003 and 2005 year.

Taken all together, seaweed components between two species showed significant difference depending on harvesting month even though the average content was similar. Therefore, *A. nodosum* in July is better source for fucoidan extraction and *F. vesiculosus* in spring is suitable for food and plant fertilizer because of larger protein content.

2.2 Introduction

The demand for brown seaweed is increasing and the harvesting area of brown seaweed is more than double compare to the one of red or green seaweeds (Barsanti and Gualtieri, 2006). *Fucus vesiculosus* and *Ascophyllum nodosum* are eatable brown seaweed growing on St-Laurence River coasts in East Canada (Rioux et al., 2007a). They are well known as an intertidal marine alga and adhere to stones (Round, 1981; Dickinson, 1963). They have been discovered in North America and Europe in the North Atlantic Ocean (Coyer et al., 2011). These brown seaweeds are valuable as foods and source of bioactive elements. The general composition of *F. vesiculosus* and *A. nodosum* in Quebec was 70% of polysaccharide, 22% of protein, 7% of minerals and 1% of lipid (Rioux et al., 2007a). The portion of general components was similar between both seaweeds.

Each component has potential industrial value. For example, nitrogen and protein concentrations in seaweed are important factors to determine commercial value of seaweed for nutrition or plant fertilizer. Lipid is useful to develop cosmetic product, and mineral is worthy for healthy food. Algae polysaccharides are widly used as food stabilizers and some possess interesting biological properties. Fucoidan is well known because of its biological activities such as anti-coagulation, anti-thrombin (Park et al., 2000; Soeda et al., 2000), anti-HIV and anti-tumor activities (Moen and Clark, 1993; Béress et al., 1993). Fucoidan is a hetero- and muco-polysaccharide (Park et al., 1997a; Park et al., 2000; Percival and McDowell, 1967). It is found in intercellular spaces throughout the tissue of brown seaweed (Park et al., 1997a). The main function of fucoidan is to prevent seaweed from being dried on exposition to the air by tides (Souchet, 2004).

Polyphenols content is an important factor to increase the seaweed value because these compounds have biological functions such as antioxidant and chelating ability. Anti-oxidative property of phenols has been related to anti-cancer and anti-inflammation by free radical oxygen removing (Holiman et al., 1996). Also, phenolic extract of seaweed inhibited α-amylase and α-glucosidase (Zhang et al., 2007; Ohta et al., 2002; Bhandari et al., 2008). The inhibition of phenols onto two enzymes can be useful to delay glucose absorption and then decrease glucose level in blood because these enzymes are involved in starch digestion.

Food having low glycemia index can reduce repetitive peak in glucose each time a food containing starch is consumed resulting in stimulation of insulin production, repetitively. This overstimulation for long time can induce insulin resistance and the type-2 diabetes. The blood glucose level of diabetic patients before meal excesses the glucose level of normal person after meal. High glucose concentration in blood increases the viscosity of blood and it decrases the flow rate of blood and accumulates precipitation in blood vessel. These phenomenoms decreases the cross-sectional area of blood vessel and interrupts the delivery of nutritions and oxygen into cells. Therefore, necrosis of cell and organics can be happened.

On the other hand, seaweed components can be variable depending on harvesting period (Sheader and Moss, 1975). The comprehension of seasonal variation of algae components is necessary to decide optimal harvesting period. Several studies were performed in order to understand the relationship between season and algae components (Chapman et al., 1977; Strömgrem, 1986; Kim et al., 1996; Fleurence, 1999; Rupérez et al., 2002; Obluchinskaya et al., 2002). Mathieson et al. (1976) reported different patterns of growth rate, length, weight of brown seaweed between *F. vesiculosus* and *A. nodosum* under the same environmental condition.

Brown seaweeds like *Fucus vesiculosus, Ascophyllum nodosum* and *Laminaria longicruris* in Quebec are growing under specific environmental condition, because St-Laurence River is covered with thick ice during winter. Some of the researches between season and algae chemical components have been performed with *Laminaria longicruris* (Anderson et al., 1981; Gagné et al., 1982; and Souchet, 2004) but not on *F. vesiculosus* and *A. nodosum.* Therefore, this study aims to investigate the correlation between the harvesting period and chemical composition (protein, lipid, ash, polysaccharide, fucoidan and phenols) of *F. vesiculosus* and *A. nodosum* respectively.

2.3 Materials and Methods

2.3.1 Sample preparation

Two species of brown algae, *Fucus vesiculosus* and *Ascophyllum nodosum,* were harvested at Île-Verte (Quebec, Canada) for 3 years; in 2002 (May, June, July, September, October), in 2003 (June, July, August, September, October) and in 2005 (May, June, August, November).Algae were milled in a Comitrol Mill with perforated plates of 24.5 and 1 mm, freeze-dried and then kept at -20℃ until use. All reagants and chemicals were purchased from Sigma-Aldrich in USA and Canada.

2.3.2 Chemical composition in seaweed

The composition of crude seaweed was analyzed using official AOAC method (1990). Moisture content was measured (AOAC, 930.04; 1990). Total nitrogen in algae was determined by nitrogen analysis equipement Leco (Leco MI, USA). A conversion factor of 6.25 was used to calculate protein content. Lipid content was quantified using Folch's method (Folch et al., 1957) and mineral content was estimated from ash (AOAC, 942.05; 1990). Polysaccharide content was calculated by the following equation; Polysaccharide content (%) = 100 – (the content (%) of protein + lipid + ash).

2.3.3 Total polyphenols content

The amount of polyphenols in algae was measured according to Slinkard and Singleton (1977) and Prior et al. (1998). Dried seaweed (0.5 g) was mixed with 10 ml of acetonitrile containing 4% of acetic acid. The mixed solution was agitated for 30 min, and then centrifuged at 13,000 g for 15 min. The supernatant (1 ml) was transferred into another glass cap-tube and mixed with 0.2N Folin-Cocialteu stock reagent (0.5 ml) and 15% w/v sodium carbonate (2 ml). This solution was filled up to 10 ml of final volume with water and then mixed. After 8 min 20 sec, OD at 765 nm was measured using an UV-spectrophotometer. For standard curve, gallic acid (10 ~ 50 ppm in water) was used.

2.3.4 Fucoidan extraction

Fucoidan was extracted as presented by Rioux et al. (2009). Dried alga was mixed with 1% (w/v) $CaCl_2$ solution (30 volumes) and then stirred for 4 hours at 85℃ at 455 ± 5 rpm by using a stirrer

RZR1 (Caframo Ltd. Canada). The supernatant was separated by centrifugation (16,887g, 20 min), and vacuum filtration on Whatman No. 4 filter. The filtered liquid was mixed with 2 volumes of 95% ethanol and 1 volume of 2% (w/v) NaCl and then stirred for 1 hour at room temperature for alcoholic precipitation of fucoidan. This solution was kept at -20℃ for 48 hours. The pellet containing fucoidan was recovered by centrifugation (16,887 g, 12 min). Then, it was resolubillized in 100 ml of fresh deionized water, and dialyzed for 48 hr by using membrane of 15 kDa (Sigma, USA) to remove minor constituents and solvents. Fucoidan was recovered by freeze-drying and preserved at -20℃ in a sealed tube to keep away from humidity. Yield of fucoidan was calculated as the percentage (%) of dry weight of seaweed.

2.3.5 Fucoidan purity

The purity of the extracted fucoidan was analyzed using HPLC combining Rezex RPM Monosaccharide 50×7.8 mm precolumn (Phenomenex, USA) and Rezex RPM Monosaccharide 300×7.8 mm (Phenomenex, USA). The system consisted of Waters 715 Ultra wisp sample processor (Millipore, USA), LKB Bromma 2150 HPLC pump (LKB, Sweden) and Water 410 differential Refractometer detector (Millipore, USA) linked to Agilent interface analogue 3590E (Agilent technology, USA) with HP Chemstation Rev. A.06.03 software. The mobile phase was 0.2 μm filtered HPLC grade water and the flow rate was 0.6 ml/min. Commercial fucoidan (98% purity) of *F. vesiculosus* (Sigma-Aldrich, USA) was dissolved in deionized water to make several concentrations (125, 250, 300, 400 and 500 ppm) for standard curve.

2.3.6 Chemical composition of fucoidan fraction

Sulphur content of fucoidan was determined by ICP-OES (inductively coupled plasma-optical emission spectroscopy) using the model Optima 4300DV (Perkin-Elmer, USA) equipped with Winlab32 software. Sulfate content was calculated using the following equation (Roger et al. 2004).

$$\text{Sulfate group (\%)} = 3.22 \times \text{Sulphur (\% wt)}.$$

The determination of nitrogen content in fucoidan was same method of section 2.3.2. The determination of uronic acid was performed using the method of Blumenkrantz and Asboe-Hansen (1973). Glucuronic acid was used as the standard 2.5 μg/ml to 20 μg/ml and absorbance was measured in triplicate at 520 nm.

2.3.7 Statistical analysis

Prism 5.0 software (USA) was used for statistical analysis of the obtained data according to months and seaweed species by Two-way ANOVA and the results are shown in Annex 1 to 5. For instance, single data per month indicated as mean value of the accumulated data during three years of investigation, and marked as mean ± SD was used for statistical analysis. In order to verify the influence of month for fucoidan content, Tukey test was performed and the significant difference was detected at $p < 0.05$.

2.4 Results and discussion

2.4.1 Protein content in seaweed

The harvesting period was a significant factor on protein content ($p<0.05$). As shown in Fig. 2-1A and Table 2-1, minimal and maximal protein contents of *F. vesiculosus* during three years of investigation were 9.1% and 13.2%. At the same period, *A. nodosum* had relatively smaller content of protein as 7.0% (min.) and 10.8% (max.). Protein content is higher in May and decreases in summer and reach a lower value that remain constant in fall. The mean quantity of protein was 10.5 ± 1.5% in *F. vesiculosus* and 8.4 ± 1.3% in *A. nodosum.* A similar behaviour has been reported by Boney (1965). This lower protein content of seaweed during summer was explained by decreased nitrogen content in water and nitrogen assimilation for increased energy consumption because of high temperature during summer (Chapman et al., 1977; Souchet, 2004; Percival and McDowell, 1967; Graham and Wilcox, 2000; Anderson et al., 1981; Park et al., 1997a; Germann et al., 1987). High protein content could allow seaweed use as food or plant fertilizer. Therefore, the best harvesting period of *F. vesiculosus* and *A. nodosum* in Quebec is May for food and plant fertilizer.

2.4.2 Lipid content in seaweed

There was no significant difference of harvesting period in lipid content for both seaweeds (Fig. 2-1B). The lipid average content of *F. vesiculosus* and *A. nodosum* was low at about 1.5 ~ 1.6% (Table 2-1). This is similar to Sánchez-Machado et al. (2004) and Park et al., (1997a) reported lipid contents lower than 2% in many species of seaweeds such as *S. polyschides, H. elongate, L. ochroleuca, U. pinnatifida, Palmaria* species, and *Porphyra* species. Low lipid content of *F. vesiculosus* and *A. nodosum* will contribute few calories to the diet as mentioned by Jurković et al. (1995).

2.4.3 Total phenols content in seaweed

The quantity of phenolic substances varied depending on season (Fig. 2-1C). Both species had similar patterns with low contents from spring to early summer followed by increasing values up to September. The phenol content of *A. nodosum* in September was higher than May and June ($p<0.05$). However, *F. vesiculosus* showed rather delayed turning point to increase phenol quantity from

August (late summer) and had maximal quantity of phenol in October/November (late autumn). Comparing two seaweeds in July, *A. nodosum* had double fold of phenol content relative to that of *F. vesiculosus*. The phenols content per dried seaweed weight (100 g) was 33.8 ~ 115.7 mg for *F. vesiculosus* and 36.1 ~ 124.8 mg for *A. nodosum*.

Many studies on polyphenols of seaweed have been performed, because polyphenols have antioxidative and radical scavenging activities (Breton et al., 2011). Polyphenols are also able to inhibit alpha-glucosidase, contributing to decrease diabetic symptom (Kim et al., 2008; Zhang et al., 2007). In addition, polyphenols from *F. vesiculosus* and *A. nodosum* were more efficient antioxidant than that of other species such as *Laminaria digitata, Palmaria palmata* and *Chondrus crispus* (Wang et al., 2009a). The amount of phenolic compounds in seaweeds was debated. For instance, the quantity of polyphenols in *A. nodosum* was reported as 0.07 ~ 0.13% by Ragan and Jensen (1977), 0.5 ~ 9.4% by Haug and Larsen (1958). For the amount of polyphenols in *F. vesiculosus*, there were many reports; as 0.01 ~ 0.06% by Ragan and Craigie (1976) and 0.08 ~ 0.17% by Ragan and Jesen (1977). Recently, Geiselman and McConnell (1981) reported that polyphenols contents of *F. vesiculosus* and *A. nodosum* were as low as 1% (dry weigth). On the other hand, the age of seaweed can be an effective factor that affects the amount of polyphenols. The polyphenols were increased by age of the tissue of *A. nodosum* (Pederson, 1984). It was also reported that large quantity of heavy metals contributed to the accumulation of the phenol (physodes) in *F. vesiculosus* (Forsberg et al., 1988). The polyphenols content of *F. vesiculosus* and *A. nodosum* in Quebec was approximately 0.2% and less. For polyphenol content (Fig. 2-1C), *F. vesiculosus* in Oct&Nov. was higher than May to August ($p < 0.05$) and *A. nodosum* in September had significantly higher than May and June ($p < 0.05$). Overall, autumn (September and October) was considered optimum seaweed harvesting period than spring for phenolic compounds extraction.

2.4.4 Mineral content in seaweed

Minerals were estimated from the ash of seaweeds (Fig. 2-1D). In Table 2-1, the mineral content of *F. vesiculosus* ranged from 23.6% to 28.0% during the same period and *A. nodosum* had 17.0% to 23.3%. The average content of minerals, during three years of investigation, was 25.3% and 20.1% for *F. vesiculosus* and *A. nodosum,* respectively. Considering of the variability, there was no significant difference between *F. vesiculosus* and *A. nodosum*. However the mineral content of *A.*

nodosum reached its lowest value in August. Ito and Hori, (1989) and Ortega-Calvo et al. (1993) reported that mineral content of seaweed is higher than that of land plants and animal products. *F. vesiculosus* and *A. nodosum* contained high mineral content similar to the reports of Mabeau and Fleurence (1993) and Ortega-Calvo et al. (1993) which were 8% to 40% of mineral content. It shows that two species brown seaweeds in Quebec can be a good source of mineral for food and health products.

According to Park (1969), the mineral content of *Ecklonia cava, Sargassum sugamianum* and *Hizikia fusiforme* increases from autumn to spring, thus mineral content in spring should be higher than autumn.

2.4.5 Polysaccharide content in seaweed

Polysaccharide content in *F. vesiculosus* ranged from 59.5% to 67.3% depending on harvest period and *A. nodosum* contained 64.0% to 73.5% of polysaccharide (Fig. 2-1E). The average polysaccharide content for 3 years, *F. vesiculosus* was 62.6% and *A. nodosum* 70.2% (Table 2-1). *A. nodosum* had slightly higher polysaccharide than *F. vesiculosus* similarly to Rioux et al.'s (2007a) which showed that the polysaccharide content of seaweeds in September 2002 was 65.7 ± 0.4% for *F. vesiculosus* and 69.6 ± 0.2% for *A. nodosum*. Results obtained for *F. vesiculosus* and *A. nodosum* also correspond with Bobin-Dubigeon et al. (1997) and the content is larger than other brown seaweed species such as *Hijiki* (49.2%), *Wakame* (35.3%) and *Himantalia elongate* (32.7%) (Jiménez-Escrig and Sànchez-Muniz, 2000). The polysaccharide content of both seaweeds was not significantly different depending on harvesting month. However, the polysaccharide content tends to increase from spring to autumn. *A. nodosum* harvested in July and August had significantly higher polysaccharide content than *F. vesiculosus* ($p<0.05$).

Numerous studies on polysaccharides of seaweeds have been done because of their various biochemical functions (Park et al., 2000; Percival and Mc Dowell, 1967; Chevolot et al., 2001). The major polysaccharides of brown seaweeds are cellulose, alginate, fucoidan and laminaran (Park et al., 2000). Cellulose has recently gain interest as a material for biofuel production (Ragauskas et al., 2006; Weng et al., 2008) and alginate is a valuable component as gelification and food ingredient (Park et al., 1997b; Rioux et al., 2007a). Fucoidan is interesting because of its biological activities (Koyanagi et al. 2003; Moen and Clark, 1993; Zemani et al., 2005). In this study, high content of

polysaccharide could be an indicator on which brown seaweeds in Quebec have high potential value in commercial industries.

2.4.6 Fucoidan

2.4.6.1 Yield and purity of crude fucoidans

In observation of influence of harvesting year, the difference of annual average content of fucoidan among 2002, 2003 and 2005 was not significant ($p > 0.05$) in both seaweeds (Table 2-2) and the result was arranged by months. Total yield of the fucoidans which were obtained from *F. vesiculosus* ranged from 2.8% to 3.4%, with an average value of 3.1 ± 0.4%. The fucoidan content of *A. nodosum* was from 2.6% to 4.1%, with an average value of 3.2 ± 0.7%. The average fucoidan content over the three years did not show significant difference between both seaweeds ($p > 0.05$). However, the influence of harvesting period was observed differently depending on seaweed species. In Fig. 2-2A, fucoidan content of *A. nodosum* in May and July respectively was higher than September and Oct&Nov ($p < 0.05$), and there was no significant difference in fucoidan content between May and July ($p>0.05$).

On the other hand, the purity of fucoidan extract was investigated by HPLC analysis. As shown in Fig. 2-2B, the crude fucoidans from both seaweeds showed a high and stable purity. There was no significant difference between two species in purity; 81.0 ± 5.9% for fucoidans of *F. vesiculosus* and 86.6 ± 4.4% for fucoidans of *A. nodosum*. Considering the purity, pure fucoidan content in July was higher than May ($p < 0.01$), therefore the best harvest month of *A. nodosum* was July to extract higher content of pure fucoidan.

Obluchinskaya et al. (2002) reported that the fucoidan content of brown seaweeds in Barents Sea showed no significant difference depending on season and *A. nodosum* had lower fucoidan content than *F. vesiculosus, F. distichus* and *F. serratus*. According to Souchet (2004), fucoidan content of *Laminaria longicruris* in Quebec was high in May, June and November, and low in July. In addition, galactofucoidan content and its monosaccharide compostion of *Saccharina longicruris* varied depending on seaweed harvesting period and extracting method (Rioux et al., 2009). The study of algae from different regions and seaweed species makes difficult their comparison.

2.4.6.2 Sulfate content in fucoidan

Sulfate content of fucoidan ranged from 17.8% to 23.4% and from 20.2% to 22.9% for *F. vesiculosus* and *A. nodosum* respectively. The fucoidan of *F. vesiculosus* showed more variability in its sulfate content depending on the month than that of *A. nodosum*. However average sulphate content of fucoidan was similar between both seaweeds at 20.3 ± 2.7% for *F. vesiculosus* and 21.5 ± 1.7% for *A. nodosum* respectively. As seen in Fig. 2-3A, the sulfate content of *A. nodosum* was not significantly different with harvest month, however fucoidan of *F. vesiculosus* in August (23.4 ± 0.8%) had higher sulphate content than that in May (18.4 ± 1.9%, $p<0.05$) and October (17.8 ± 2.0%, $p<0.01$) significantly. It shows that sulfate content of fucoidan can be different depending on the harvesting month of *F. vesiculosus*, whereas *A. nodosum* was fairly consistent.

2.4.6.3 Protein content in fucoidan

The presence of protein in fucoidan is regarded as disadvantage because it could be strongly combined with polysaccharide in general. Interestingly, the protein contents in purified fucoidans of two seaweeds were different as shown in Fig. 2-3B. The fucoidans of *F. vesiculosus* had higher content of protein than those of *A. nodosum* for all months. On the average, the fucoidan of *F. vesiculosus* contained 5.0 ± 0.9% proteins while *A. nodosum* had 3.0 ± 0.7% protein content in its fucoidans. Both seaweeds had a large content of protein in its fucoidan in May/June, however, they had relatively less protein in July.

2.4.6.4 Uronic acid in fucoidan

The fucoidan of *A. nodosum* contained more uronic acid than that of *F. vesiculosus* ($p < 0.05$) (Fig. 2-3C). While 1 g of fucoidan extracted from *F. vesiculosus* contained 26.5 ± 8.3 μg of uronic acid on average, 1 g of fucoidan extracted from *A. nodosum* had 36.0 ± 5.5 μg of uronic acid. In addition, the fucoidan of *A. nodosum* showed stable ratio of uronic acid, and there was no seasonal effect. However, *F. vesiculosus* had approximately 40% decreased uronic acid in its fucoidan from June to August.

The optimal conditions (for example, seaweed species and harvesting period) can be chosen to increase yield of seaweed products, depending on application, either for food or for specific substance. Determination of the optimal condition to use *F.vesiculosus* and *A.nodosum* in Quebec is a

more significant factor than other regions because seaweed harvesting is interrupted by water freezing during winter.

2.5 Conclusion

Annual average content of fucoidan was not significantly different with seaweed harvest year for both seaweeds. For fucoidan extraction, *A. nodosum* was better source than *F. vesiculosus* and the best period to harvest *A. nodosum* was in July. Considering of sulfate content of fucoidans, it is supposed that the functional activity of fucoidans between *F. vesiculosus* and *A. nodosum* will be similar because sulfate is well-known factor of fucoidan's biological function.

The seaweeds were composed of polysaccharide > minerals > protein > lipid > phenols in order. *A. nodosum* contained more polysaccharide than *F. vesiculosus*. In a view of protein and mineral content, it is estimated that *F. vesiculosus* and *A. nodosum* in Quebec showed a good nutritional value. *F. vesiculosus* was more suitable for food and plant fertilizer than *A. nodosum* because of its higher protein and mineral content. This study showed clearly the importance of seaweed harvest timing. Even though average content of components between seaweeds is similar, the component content varies with harvesting month.

Acknowledgment

Funding of this study was provided by CORPAQ. Authors would like to thank ABK-Gaspésie Inc. for seaweeds harvesting. They would like to thank MAPAQ for seaweeds grinding and technical assistance.

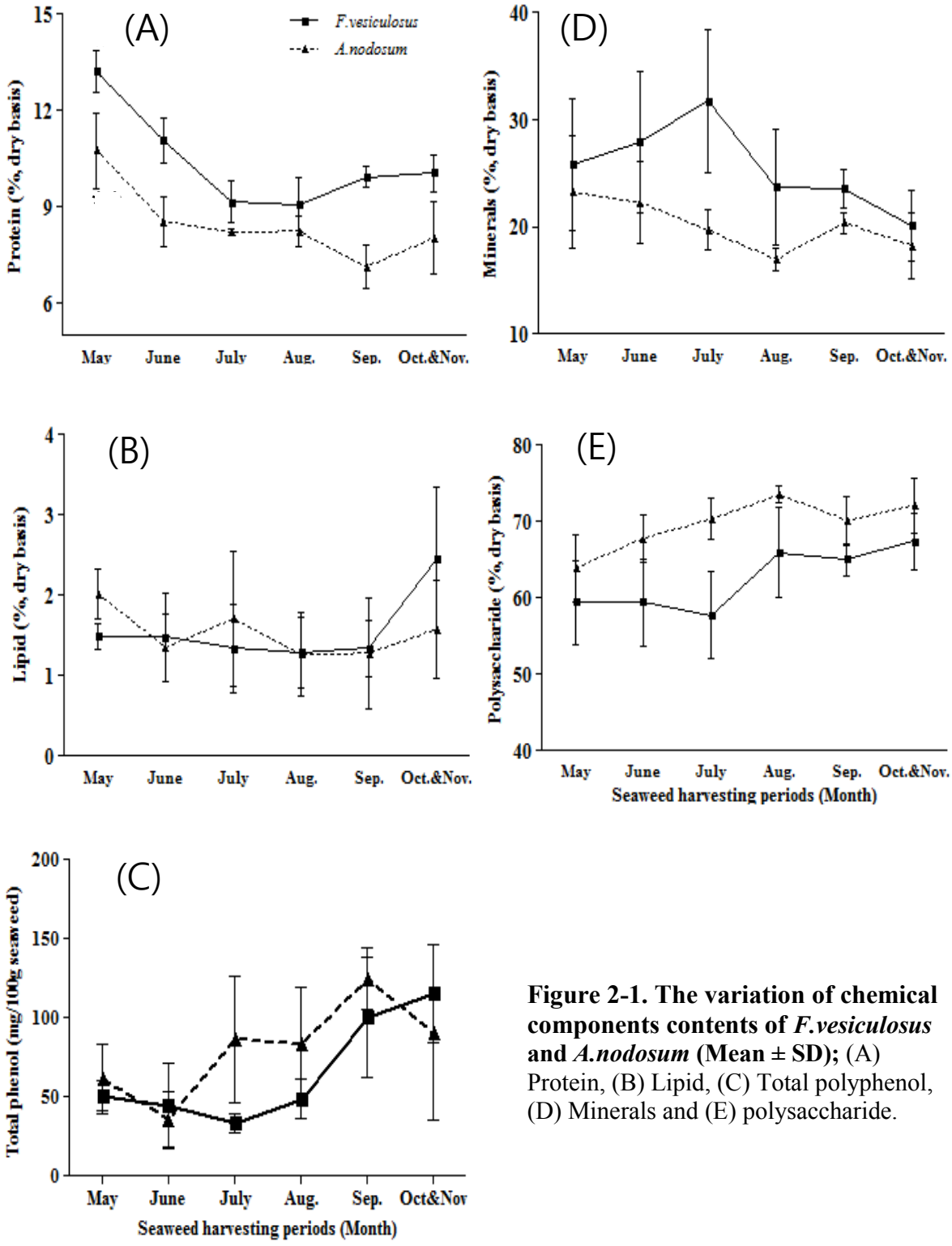

Figure 2-1. The variation of chemical components contents of *F.vesiculosus* and *A.nodosum* (Mean ± SD); (A) Protein, (B) Lipid, (C) Total polyphenol, (D) Minerals and (E) polysaccharide.

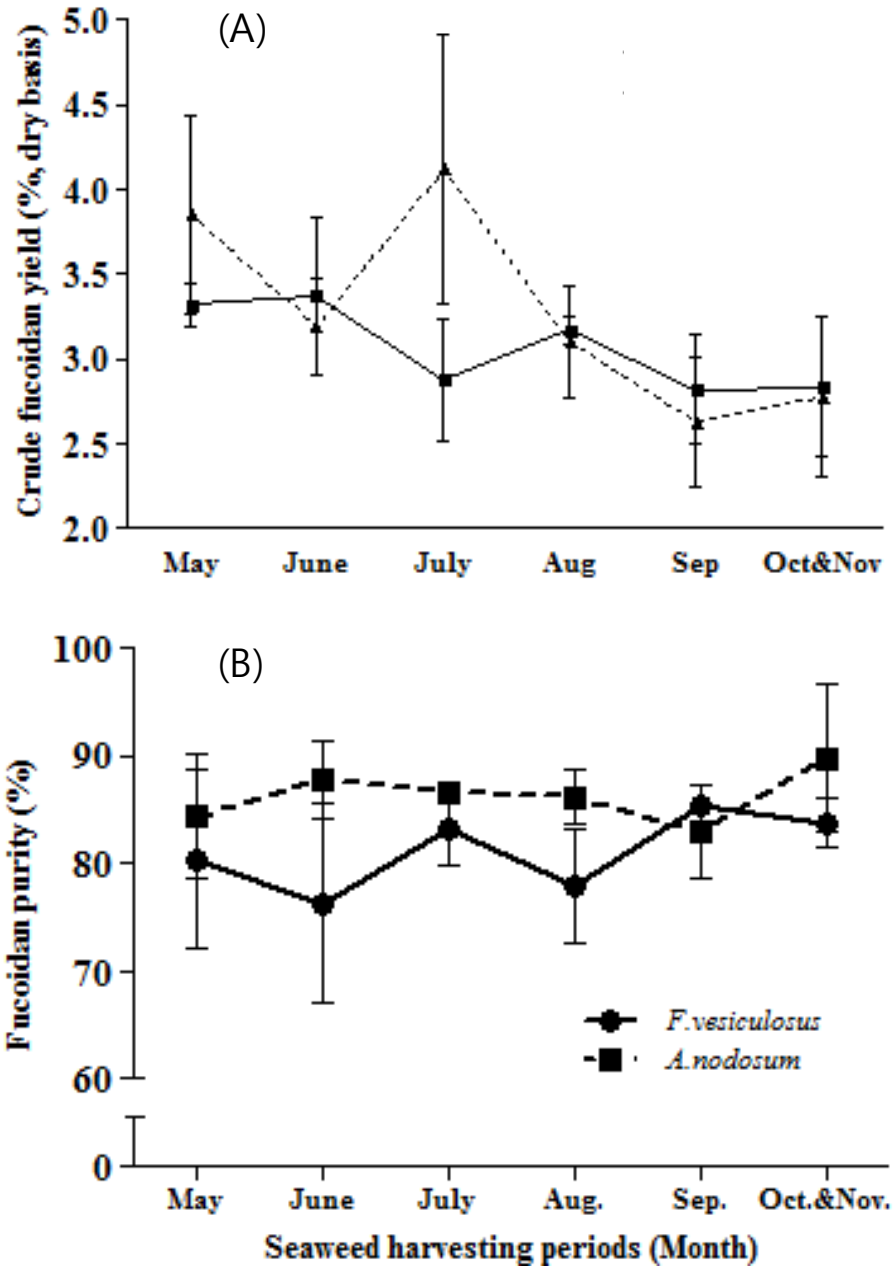

Figure 2-2. The variation of quantity and purity of fucoidans extracted from *F. vesiculosus* and *A. nodosum* (Mean ± SD); (A) Yield of fucoidan extracts, and (B) Purity of fucoidan extracts.

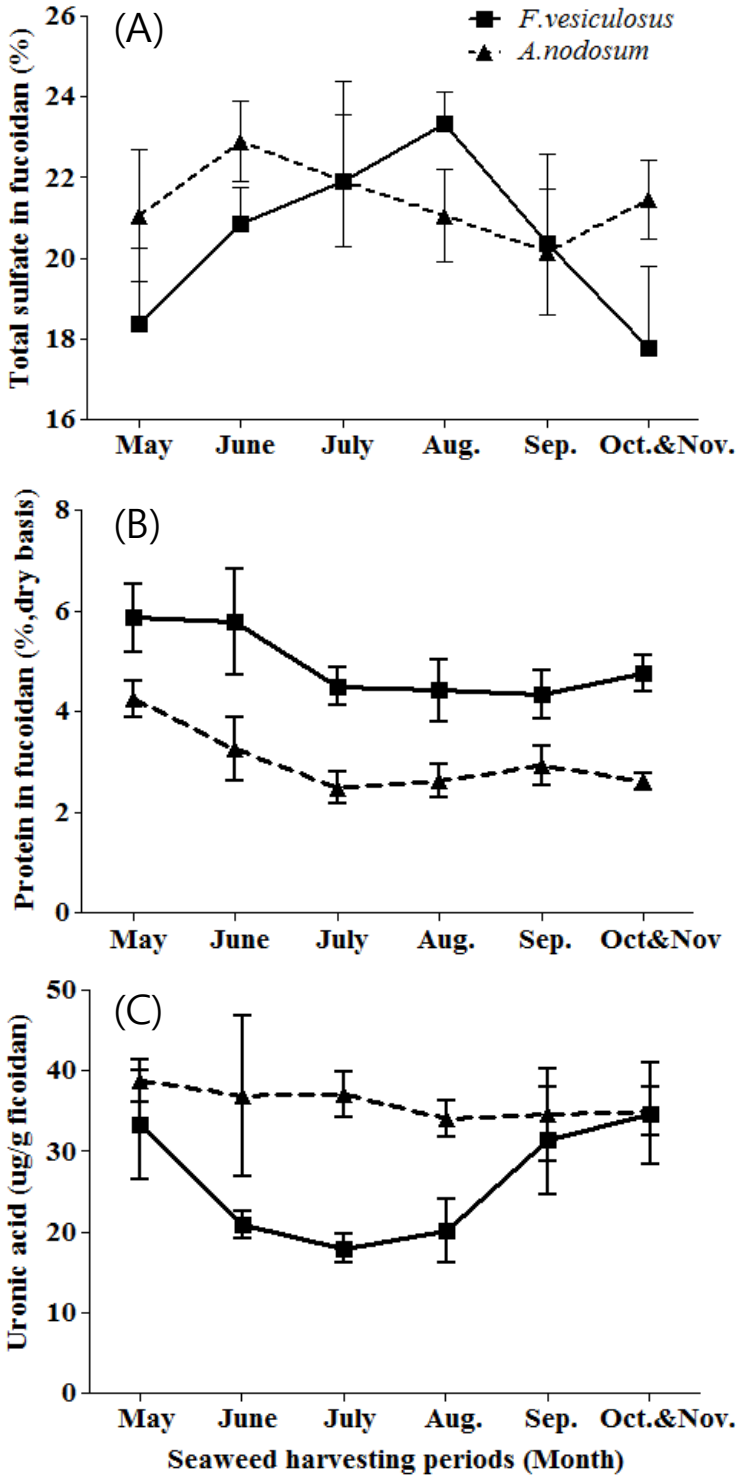

Figure 2-3. The variation of chemical components of fucoidans extracts of *F. vesiculosus* and *A. nodosum* (Mean ± SD): (A) Sulfate contents in fucoidan, (B) Protein contents in fucoidan, and (C) Uronic acid in fucoidan.

Table 2-1. Maximal, minimal and average quantity of seaweed components of *F. vesiculosus* and *A. nodosum*

Seaweed components (%)		*F. vesiculosus*	*A. nodosum*
Protein	Max.	13.2 ± 0.8	10.8 ± 1.5
	Min.	9.1 ± 1.0	7.0 ± 0.9
	Average	10.5 ± 1.5	8.4 ± 1.3
Lipid	Max.	2.5 ± 1.0	2.0 ± 0.4
	Min.	1.3 ± 0.4	1.3 ± 0.6
	Average	1.6 ± 0.7	1.5 ± 0.6
Ash	Max.	31.8 ± 8.1	23.3 ± 6.6
	Min.	20.1 ± 3.8	17.0 ± 1.4
	Average	25.3 ± 6.3	20.1 ± 3.7
Polysaccharide	Max.	67.3 ± 4.3	73.5 ± 1.3
	Min.	57.7 ± 7.0	64.0 ± 5.5
	Average	62.6 ± 6.0	70.2 ± 4.3

Table 2-2. Analysis of variance and Tukey test for fucoidan content

Covariate: Year	*A. nodosum*	*F. vesiculosus*
Degree of freedom	2	2
F - value	2.978	0.08235
Pr > *F*	0.09626	*0.9215*
Are means signif. different? (P < 0.05)	**NS***	**NS***

Chapter 3. Alpha-amylase and alpha-glucosidase inhibition is differentially modulated by fucoidan obtained from *Fucus vesiculosus* and *Ascophyllum nodosum.*

Kyung-Tae Kim, Laurie-Eve Rioux and Sylvie L. Turgeon

To be submitted to *Phytochemistry*

3.1 Abstract

Fucoidan is a water-soluble, negatively charged polysaccharide abundant in brown marine algae and many biological functions were reported for this polysaccharide. However, the inhibition of α-amylase and α-glucosidase by two fucoidan (*Ascophyllum nodosum* and *Fucus vesiculosus*) harvested at different period (months and years) has never been investigated. Fucoidans inhibited α-glucosidase depending on algal species and season of harvest. Fucoidans extracted from *A. nodosum* were a more potent inhibitor of α-glucosidase with IC_{50} ranging from 0.013 to 0.047 mg/mL than those from *F. vesiculosus* (IC_{50}= 0.049 mg/mL). In contrast, fucoidan extracted from *F. vesiculosus* did not inhibit α-amylase, while, fucoidan from *A. nodosum* decreased α-amylase activity by 7 to 107% at 5 mg/ml, depending the algae harvest period. An IC_{50} of 0.12 to 4.62 mg/mL for fucoidan from *A. nodosum* was found. The ability of fucoidan to inhibit α-amylase and α-glucosidase varies according to algae species and harvest period. Thus, *A. nodosum* is more suitable than *F. vesiculosus* as a source of fucoidan to reduce glucose production by inhibiting α-amylase and α-glucosidase activity.

Keywords: Fucoidan, α-amylase, α-glucosidase, inhibition*, Fucus vesiculosus, Ascophyllum nodosum,* Type-2 diabetes, season.

3.2 Introduction

Diabetes is a metabolic disorder characterized by high plasma glucose levels (Mitrakou et al., 1992; Daniel et al., 2001) Diabetes is classified as Type-1 and Type-2. Type-1 or insulin-dependent diabetes is due to failure of the pancreas to secrete insulin, while Type-2 or non-insulin-dependent diabetes is the result of insufficient insulin production. Wild et al. (2004) estimated that 246 million persons in the world suffered from Type-2 diabetes in 2007 and that this number will reach at least 380 million by 2025.

Type-2 diabetes receives more attention than Type-1 because it is considered avoidable. Type-2 diabetes is caused by imbalance between blood sugar absorption and insulin secretion. Post-prandial hyperglycemia plays an important role in development of Type-2 diabetes (Baron, 1998). The control of plasma glucose level is essential to delay and even prevent Type-2 diabetes. To reach this goal, increasing or stimulating insulin secretion through medication (Wang, 1998; Goldberg et al., 1998; UK Prospective Diabetes Study Group, 1998; Daniel et al., 2001) and/or by dietary supervision could be achieved. Dietary control is suggested as a safe and complementary treatment of diabetes. A diet based on glycemic index is currently one of the most recommended nutritional treatments. It has been reported that dietary therapy can be used simultaneously with other medical treatments in order to obtain a synergistic effect (Jenkins, 1981; Wolever et al., 1994; Franz, 2000; Ganon et al., 2001). However, this has the drawback of limiting the types and quantity of food consumed. Another possible solution is to decrease the rate of blood sugar absorption from the small intestine by slowing and interrupting the digestion of dietary starch, the major source of glucose (Zhang et al., 2007; Bhandari et al., 2008; Ali et al., 2006; Dolečková-Marešová et al., 2005). This approach is considered more efficient than controlling insulin secretion, for economic reasons, convenience and avoidance of side effects (Porte, 2001) The inhibition of enzymes that digest dietary starch into glucose, α-amylase and α-glucosidase, has been studied as a way of controlling blood sugar level (Svensson et al., 2004; Ali et al., 2006; Geng and Bai, 2008). α-Amylase from human saliva catalyses the hydrolysis of α-(1,4)-glucosidic linkages and produces maltose and glucose from starch (Søgaard et al., 1993; Teeri, 1991), while α-glucosidase releases glucose from maltose (Roth et al., 2003; Mohan and Pinto, 2007). By inhibiting these two enzymes, the absorption of glucose into the bloodstream can be delayed and thus, ameliorating Type-2 diabetes symptoms

like hyperglycemia. Attempts have been made to identify α-amylase and α-glucosidase inhibitors that can be used as food or food additives. Although Seo et al. (2005) and Kato et al. (2005) demonstrated that some sugar-like phenolic compounds have α-glucosidase inhibitory activity, most studies on α-amylase and α-glucosidase inhibitors have focused on the utilization of proteins or phenolic compounds (Kim et al., 2000; Song et al., 2005; Zhang et al., 2007; He et al., 2006 (a&b); Bhandari et al., 2008; Lee et al., 2008). Although phenolic compounds have high inhibitory activity of α-amylase and α-glucosidase, phenolic compounds are unstable, sensitive to light and heat treatment limits their uses as nutraceuticals.

Recently, algae have been considered as a source of enzyme inhibitors. As several plant extracts, algae contain some polyphenolic compounds as bromophenols (Kurihara et al. 1999, Liu et al. 2011), phlorotannins (Zhang et al. 2007, Nwosu et al. 2011) which are inhibitors of α-glucosidase. Also, polysaccharides, isolated from algae, have become attractive in the biomedical area for numerous bioactivities (Gupta and Abu-Ghannam, 2011; Holdt and Kraan, 2011; Li et al., 2008). In this context, fucoidan a polysaccharide found in brown algae and having several bioactivities appears promising. Fucoidan is abundant in brown seaweed such as *Fucus vesiculosus* and *Ascophyllum nodosum* (Percival and McDowell, 1967). It is a mucilaginous, hygroscopic and sulfated polysaccharide (Black, 1954) and it protects seaweed from dehydration (Percival and McDowell, 1967). The biological effects attributed to fucoidan are: anti-coagulant (Church et al., 1989; Boisson-Vidal et al., 2000), anti-HIV (Moen and Clark, 1993; Schaeffer and Krylov, 2000) and anti-tumor (anti-angiogenesis) activities (Koyanagi et al., 2003). Brown algae contain up to 10% of fucoidan and the quantity varies depending on the region, species and season (Mabeau et al., 1990; Park et al., 1997a). The structural and chemical characteristics of fucoidan also vary among algal species (Bilan et al., 2002; Pereira et al., 1999; Chevolot et al., 2001; Marais and Joseleau, 2001).

The aim of this study was to investigate the inhibition of starch digestive enzymes (α-amylase and α-glucosidase) by fucoidan extracted from *F. vesiculosus* and *A. nodosum* harvested in Eastern Canada over several years and months.

3.3 Material and methods

3.3.1 Algae and chemicals

The brown marine algae *F. vesiculosus* and *A. nodosum* were harvested near L'Isle Verte (latitude 47° 48.6' N and longitude 69° 33.0' W) from the St. Lawrence River in Quebec (Eastern Canada) from May to November in 2002, 2003 and 2005. Algae were milled in Comitrol Mill fitted with perforated plates of 24.5 and 1 mm, freeze-dried and then kept at -20 ˚C until use. Cornstarch used as a substrate for α-amylase assay (Novation 9230 organic corn starch) was provided from National Starch Food Innovation Canada. Water used in the experimental procedures was distilled de-ionized water and filtered using 0.2-µm membrane. All chemicals were purchased from Sigma-Aldrich (ON, Canada)

3.3.2 Fucoidan extraction

Lyophilized algae mass (15 g) was suspended in 450 mL of 1% $CaCl_2$ solution and stirred for 4 h at 85˚C using an RZR1 stirrer (Caframo Ltd. Canada) set at 455 ± 5 rpm. After centrifugation (16,887 g, 12 min), the liquid phase was separated by vacuum filtration using Whatman No. 4 filter paper. The filtrate was mixed with two volumes of 100% ethanol and one volume of 1% NaCl solution. The mixture was kept at -20 ˚C for 48 h. The precipitates were collected by centrifugation (16,887 g, 20 min) and completely re-suspended in 100 mL of de-ionized water. This solution was dialyzed for 48 h using a 15 kDa cut off dialysis membrane (Fisher Sci., USA). The dialyzed fucoidan was freeze-dried and kept at -20 ˚C in sealed tubes. Two extractions were conducted per seaweed sample.

3.3.3 Alpha-amylase inhibition assay

The method of Conforti et al. (2005) was modified to determine the inhibitory effects of the fucoidan extract on α-amylase (from human salivary, EC 3.2.1.1). A 1% starch solution was prepared by stirring 1 g of corn starch in 100 mL of 20 mM sodium phosphate buffer with 6.7 mM sodium chloride, pH 6.9. The solution was heated at 100˚C for 15 min and then cooled to room temperature. The volume was brought to 100 mL with distilled water. An α-amylase (A0521, Sigma, On, Canada) solution was prepared as 1 unit/mL. The colorimetric reagent was prepared as shown in the method of Conforti et al. (2005). The fucoidan solution (0.1 mL) was

added in 1 mL of a starch solution to give final concentrations of 0, 0.05, 0.1, 0.5, 1.0 and 5 mg/mL. The tubes were prepared in duplicate and split into two groups, which were classified as a test group (TG) and a control group (CG). All tubes were incubated at 20 ˚C for 10 min, and then, 1 mL of α-amylase solution was added. For amylase activation, the TG tubes were incubated at exactly 20˚C for 5 min. The colorimetric reagent (1 mL) was added to all of the tubes of the TG, heated at 100 ˚C for 15 min and then cooled in an ice bath. 9 ml of distilled water was added to each tube, and the absorbance at 540 nm was measured. The same steps were realized for the CG, but the incubation at 20°C for 5 min was omitted. As blank, 0.1 mL of distilled water was added instead of the fucoidan solution (0 mg/mL fucoidan). The optical density of the blank (OD_B) refers to the difference between test and control group at 0 mg/ml of fucoidan. The degree of enzyme inhibition was calculated using the following equation:

$$\text{Enzyme inhibition (\%)} = \frac{(OD_{TG} - OD_{CG}) - OD_B}{OD_B} \times 100(\%)$$

3.3.4 Alpha-glucosidase inhibition assay

For the determination of α-glucosidase (EC 3.2.1.20) inhibitory activity of fucoidan, all solutions were prepared according to the method of Halvorson and Ellias (1958). 5 ml of 67 mM potassium phosphate (pH 6.8 at 37 ˚C) and 0.2 mL of 3 mM glutathione solution were added into total 10 test-tubes. They were split into two groups: the test group (TG) and control group (CG). 0.2 mL of 1 unit/mL of α-glucosidase (G5003, Sigma) was added only into TG tubes and 0.2 mL of distilled water was added into CG tubes instead of α-glucosidase. 0.1 mL of fucoidan solutions were added to get a final concentration of 0, 0.005, 0.01, 0.025 and 0.05 mg/mL in both TG and CG tubes in parallel. As blank, 0.1 mL of distilled water was added instead of the fucoidan solution (0 mg/mL fucoidan). The optical density of blank (OD_B) refers to the difference between test and control group at 0 mg/ml of fucoidan. All tubes were kept at 37 ˚C for 20 min. 0.5 mL of 10 mM p-Nitrophenyl alpha-D-glucopyranoside was added into all tubes and then incubated at 37 ˚C for 20 min. 1 ml of the previous mixture solution was transferred into new test tube containing 4 mL of sodium carbonate (0.1 M), and the solution

was mixed. The absorbance was measured at 400 nm and the degree of enzyme inhibition was calculated using the following equation:

$$\text{Enzyme inhibition (\%)} = \frac{(OD_{TG} - OD_{CG}) - OD_{B}}{OD_{B}} \times 100(\%)$$

3.3.5 Statistical analysis

Enzyme inhibition analyses were performed in duplicate with two repetitions for each fucoidan sample. Results are presented as mean ± standard deviations (SD) for each harvested period. Significant differences were found as when $p < 0.05$. Statistical analysis was carried out using Prism version 5.0 (GraphPad Software, San Diego, USA) for the IC_{50} calculation. The enzyme activity data were analyzed using SAS 9.2 software (SAS Institute Inc., Cary, USA). To determine the effect of fucoidan purity on the enzyme activity, the purity of each fucoidan sample was added to the statistical model as a covariate in the mixed model analysis. Since the year when fucoidan was harvested was significant at $p < 0.05$ another test was realized for each year at the higher fucoidan concentration to compare the least squares means of months using Tukey adjustment with $p < 0.05$.

3.4 Results and discussion

Most studies looking for natural extracts contributing to diabetes prevention have been focusing on α-glucosidase inhibition because this enzyme plays a role at the ending step of starch digestion by producing glucose from maltose. Although the inhibition of α-amylase also results in the decrease of glucose release, its complete inhibition is not desired because it could provoke intestinal disorders (Cho et al., 2011a). The undigested starch could be utilized by the gut microflora for gas production. Therefore, partial inhibition of α-amylase could contribute to modulate the rate of glucose release from starch.

3.4.1 Inhibition of α-amylase by fucoidan

In this study, brown algae harvested from Eastern Canada from May to November in 2002, 2003 and 2005 were used to determine the ability of fucoidan to inhibit α-amylase throughout different harvest period. No harvesting was possible during winter because of ice. The range of fucoidan concentrations investigated (0 to 5 mg/mL) was established by preliminary tests.

Interestingly, α-amylase inhibitory activity of fucoidan was completely different depending on the seaweed source. The fucoidans extracted from *A. nodosum* significantly suppressed α-amylase activity depending on harvest periods and fucoidan concentrations. In contrast, none of the fucoidan extracts from *F. vesiculosus* inhibited α-amylase activity in the range of concentration studied (data not shown). This difference indicates that the source of fucoidan has a strong influence on its inhibitory capacity. It should be noted that the same extraction method was used for both seaweed species from which fucoidan was extracted and they were similar in general composition, as shown previously (Fig 2-2B in Chapter 2).

Several concentrations of fucoidan were tested (0.05 to 5 mg/mL). Statistical analysis revealed that the purity of fucoidan fractions did not impact their activity (average purity: 87% for *A. nodosum* and 81% for *F. vesiculosus*). At low dose of fucoidan (0.05 mg/mL), no significant effect was observed for the α-amylase activity (Table 3-1). As the concentration increased (0.1 to 5 mg/mL), the independent variables (year and/or months) and the interaction of those

variables explain most of the variation observed. α-Amylase activity induced by fucoidan (5 mg/mL) for each harvest period is shown in Figure 3.1. Although we don't see any clear trends for the α-amylase activity, the inhibition is significantly lower in May 2002 and 2005 (20% inhibition) and no inhibition was observed in 2003. Late summer months present generally significant higher inhibition activity (> 83% in October 2002 and in August 2005). The results indicate important variation according to the harvest period. An IC_{50} value ranging from 0.12 to 4.62 mg/mL was found (Table 3-2). According to Black (1954), the ratio of L-fucose in fucoidan obtained from *F. vesiculosus* and *A. nodosum* varies depending on the harvest period. The seasonal variation of fucoidan structure has not been reported for those seaweeds. But others have demonstrated for other brown seaweeds that the structure changes according to the harvest period (Skriptsova et al., 2010; Rioux et al., 2009). The variation in α-amylase inhibitory activity induced by fucoidan may depend on the fucoidan structure (sulfate, uronic acid) which was previously shown to vary according to the harvest period.

The ability of fucoidan to inhibit α-amylase has been reported by Cho et al. (2011a) using an extract from *Undaria pinnatifida* (wakame). Native fucoidan fraction did not show any inhibition activity but an oversulfated fucoidan slightly reduced the enzyme activity. This study introduced a possible relationship between sulfate content of fucoidan and its α-amylase inhibition capacity. In Cho's et al. (2011a) studies, a sulfate content as high as 51% was required to inhibit α-amylase compared to 42% for native fucoidan. However, all fucoidan extracts from *A. nodosum* which showed α-amylase inhibition were less sulfated than *U. pinnatifida* fucoidan (< 25% of sulfate content). In addition, the average sulfate content of fucoidan was not significantly different between *F. vesiculosus* (20.3%) and *A. nodosum* (21.5%) (data not shown). The results of α-amylase inhibition by fucoidan in this study are not in agreement with the result of Cho et al. (2011a). It should be considered that other structural features might be involved in α-amylase inhibition.

3.4.2 Inhibition of α-glucosidase by fucoidan

Several concentrations of fucoidan were tested (0.005 to 0.05 mg/mL) to determine α-glucosidase inhibition. Statistical analysis revealed that the purity was significant only for *F. vesiculosus* at 0.01 mg/mL. At low dose of fucoidan (0.005 and 0.01 mg/mL), the independent

variation does not completely explain the variation in α-glucosidase activity (Table 3-3). As the concentration is increased, most of the variation is explained by the independent variables (year and months) which mean the α-glucosidase activity is significantly different throughout each seaweed harvest period. Results presenting α-glucosidase activity inhibited by fucoidan (0.05 mg/mL) is shown in Figure 3-2 and 3-3. Fucoidans from both *F. vesiculosus* and *A. nodosum* inhibited α-glucosidase activity. Inhibition depended on both the harvesting period and the algal species, being greater for *A. nodosum*. For *F. vesiculosus* the maximal inhibition capacity was 51.4% (September 2002) while for *A. nodosum* the inhibition was 99.6% (October 2002 and 2003). Even at a concentration of 0.025 mg/mL, fucoidan extracted from *A. nodosum* in autumn (October/November) showed remarkable α-glucosidase inhibition (over 80%). The IC_{50} values for α-glucosidase inhibition are shown in Table 3-4 using fucoidan obtained from *A. nodosum*. Results showed that the IC_{50} ranged from 0.013 to 0.047 mg/mL for *A. nodosum*. On the other hand, IC_{50} value for the fucoidan of *F. vesiculosus* could only be calculated for October 2002 (IC_{50}= 0.049 mg/mL) because it is the only harvest period that reached an inhibition of 50% at the concentrations tested. In overall, fucoidans from *A. nodosum* showed better α-glucosidase inhibition than those of *F. vesiculosus* and the highest inhibitory activity was obtained in the autumn (October/ November) for *A. nodosum*.

The IC_{50} of the fucoidan obtained from *A. nodosum* ranged from 0.013 to 0.047 mg/mL making it more potent than other α-glucosidase inhibitors including acarbose (IC_{50} = 1 mg/mL), which is used as medication for Type-2 diabetes (Table 3-5). It is also lower than the IC_{50} values of several plant extracts as tea and other food products which IC_{50} range from 11.1 to 519.8 mg/mL (Table 3-5). In addition, the IC_{50} of fucoidan from *A. nodosum* was lower than polyphenols extracted from *A. nodosum* having IC_{50} values ranging from 0.024 to 0.077 mg/mL (Zhang et al., 2007). These authors also studied the effect of a polyphenol fraction and a polysaccharide enriched fraction included in the diet of diabetic mice (200 mg/kg body mass of extracts). While the polyphenol fraction reduced rapidly glucose level after a 14 days diet, the polysaccharide enriched fraction did not decrease it significantly. Apostolidis and Lee (2010) reported a strong α-glucosidase inhibition (IC_{50} 0.24 µg of fresh algae) and a mild α-amylase inhibitory effect (1.34 µg of fresh algae) with a water-soluble extract from *A. nodosum*. However, they did not mention the fucoidan content in the extract. In our study, fucoidans from *F. vesiculosus* and *A. nodosum* in October 2002 contained about 0.1% of polyphenols.

Nevertheless, it is considered that α-amylase and α-glucosidase inhibition by fucoidan is not due to the contamination by phenolic compounds. At the maximal dose (0.05 mg/mL), only 0.05 µg of polyphenol is recovered with fucoidan which is five time less than the IC_{50} found for α-glucosidase (Apostolidis and Lee, 2010). For the α-amylase, 5 mg of fucoidan contained 5 µg of polyphenol which could be enough to inhibit the enzyme according to Apostolidis and Lee (2010). However, only *A. nodosum* has inhibited the enzyme while no activity was observed for *F. vesiculosus*. In addition, Zhang et al. (2007) used a protocol similar to ours but omitting the $CaCl_2$ step to extract polysaccharide which we believe is fucoidan and alginate. After feeding diabetic mice with polyphenol or a polysaccharide during 14 days, only the polyphenol fed group was significantly different. This indicates that the 200 mg/kg polysaccharide diet does not contain enough polyphenol to modify the fasting serum glucose. Thus, it is unlikely that the amount of residual polyphenol found in fucoidan would have interfered with our results. Also, we believe the reason why the polysaccharide fraction did not improve fasting serum glucose in Zhang et al. (2007) study is because of the presence of alginate which is soluble in water when no calcium chloride is used.

Mechanisms of α-glucosidase inhibition differ among the various inhibitors reported. Some well-known inhibitors as acarbose mimic the enzyme substrate (Seo et al. 2005). Previously published articles on the mechanism of polyphenol compounds suggest that the principal factor acting on α-glucosidase activity is hydrogen scavenging because α-glucosidase provides hydrogen to catalyze the hydrolysis of the α-(1,4)-glucosidic linkage (Braunt et al., 1995; Borges de Melo et al., 2006; Mohan and Pinto, 2007). The inhibitor acts by intercepting the hydrogen ion freed from the α-glucosidase catalytic site. Most studies conducted with fucoidan showed that the sulfate content is closely related to its biological properties. Wang et al. (2009b) showed a relation between sulfate content and radical scavenging property using fucoidan from *Laminaria japonica.* So et al. (2007) also reported free-radical scavening ability of fucoidan from *F. vesiculosus*. Highly sulfated fucoidan (37% sulfate groups) showed better free radical scavenging activity than native fucoidan (28% sulfate groups). Increasing sulfate groups may enhance the scavenging activity of fucoidan and thus, promote its capacity to intercept free hydrogen. Numerous phenolic compounds such as flavonol (Lee et al., 2008; Gamberucci et al., 2006), catechins and theaflavins (Matsui et al., 2007; Bhandari et al., 2008) have an α-glucosidase inhibitory activity. The intensity of the activity is related to the structure

and conformation of polyphenols (Matsui et al., 2007). Fucoidan might inhibit α-glucosidase by a mechanism similar to that of polyphenols that involves scavenging but more research will be needed in order to determine the mechanism of action. Since the inhibition of α-amylase and α-glucosidase varies in a great manner between both seaweed sources, we believe that the structure of both kind of fucoidan differs. Structural features such as molecular weight and the amount of sulfate groups could seriously impact the enzyme activity. More work was realized in order to explain how those structural characteristics influence the enzyme activity and this is discussed in Chapter 4.

Finally, when comparing the inhibition of the two digestive enzymes studied, α-amylase and α-glucosidase, it should be emphasized that at least 100 times more fucoidan is needed (5 mg/ml) to inhibit α-amylase to the same degree as α-glucosidase (0.05 mg/mL). This difference in inhibition offers a complementary effect because as reported by Cho et al. (2011a), high α-amylase inhibition could be related to intestinal discomfort, so a moderated inhibition of α-amylase and a stronger glucosidase inhibition would be preferred. The level of inhibition obtained with fucoidan should have the desired effect by slowing the absorption of glucose from the intestine into the bloodstream enough to allow the insulin-based mechanism of the diabetic patient to transport blood sugar into the muscles before it can reach harmful levels. These data overall suggest that fucoidan could be useful as a component of new medication, as a food additive or as a food supplement for an enzyme-targeted treatment of Type-2 diabetes. More information on fucoidan structural features are required to understand the mechanism by which fucoidan inhibits α-amylase and α-glucosidase and why fucoidan obtained from *A. nodosum* is the only one to inhibit α-amylase.

3.5 Conclusion

This study has revealed a novel function of fucoidan as an efficient inhibitor of the starch-digesting enzymes α-amylase and α-glucosidase. In summary, the enzyme-inhibiting activity of fucoidan was quite variable, depending on the algal species from which the fucoidan was extracted, the month and year during which the algae were harvested and the targeted enzyme (α-amylase or α-glucosidase). Not all fucoidans are α-amylase inhibitors. Fucoidan from *A. nodosum* inhibited both α-amylase and α-glucosidase but the required quantities were much higher for α-amylase inhibition. The best harvesting period for fucoidan potency was summer/autumn in the case of *A. nodosum* for α-amylase inhibition and autumn for both *A. nodosum* and *F. vesiculosus* for α-glucosidase inhibition. Harvested in Eastern Canada, *A. nodosum* has greater potential for the prevention of Type-2 diabetes than *F. vesiculosus*.

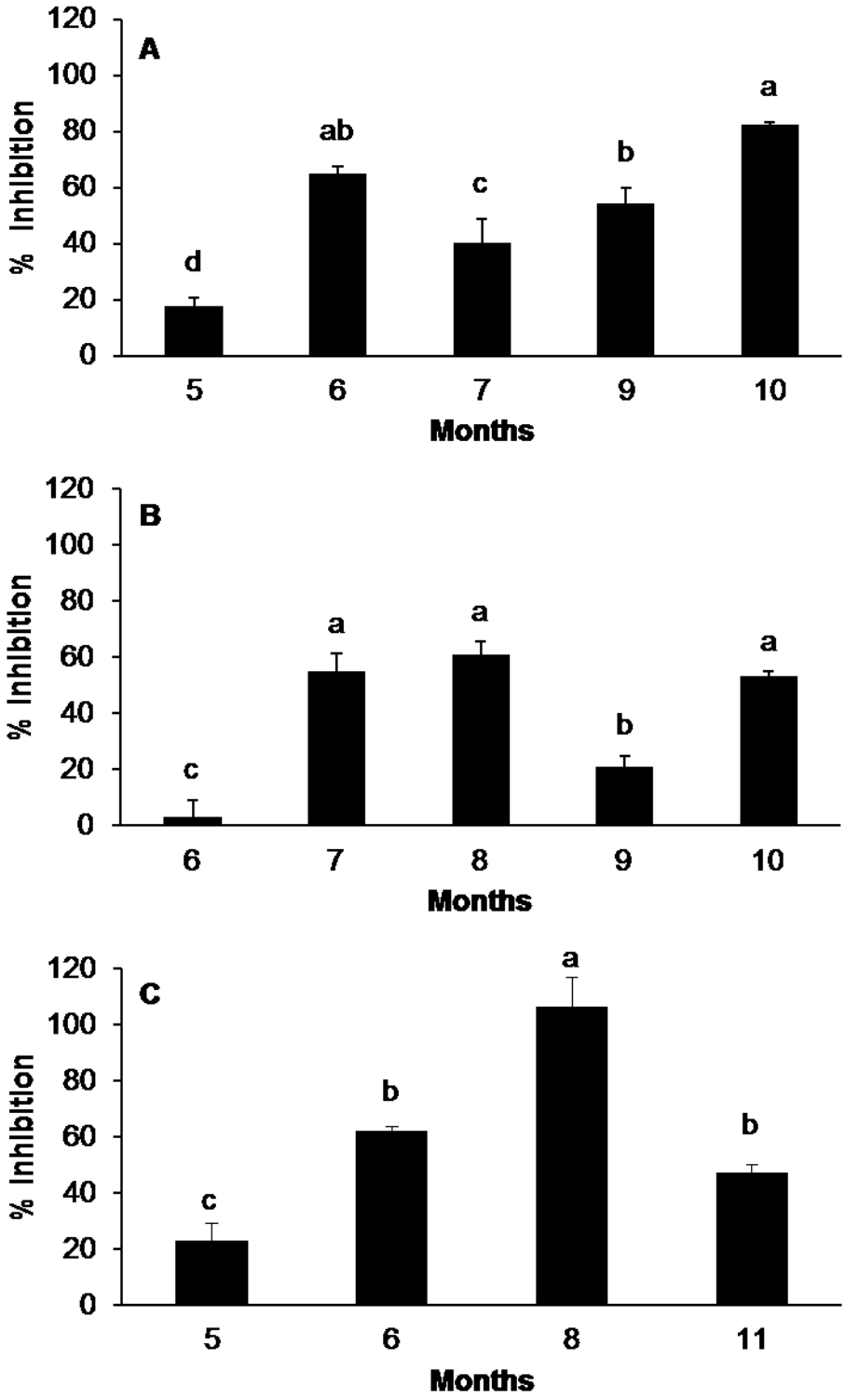

Figure 3-1. α-Amylase inhibitory activity induced by fucoidans (5 mg/mL) extracted from *A. nodosum* harvested in: A-2002; B-2003 and C-2005. Identical letters within the same panel are not significantly different at $p < 0.05$.

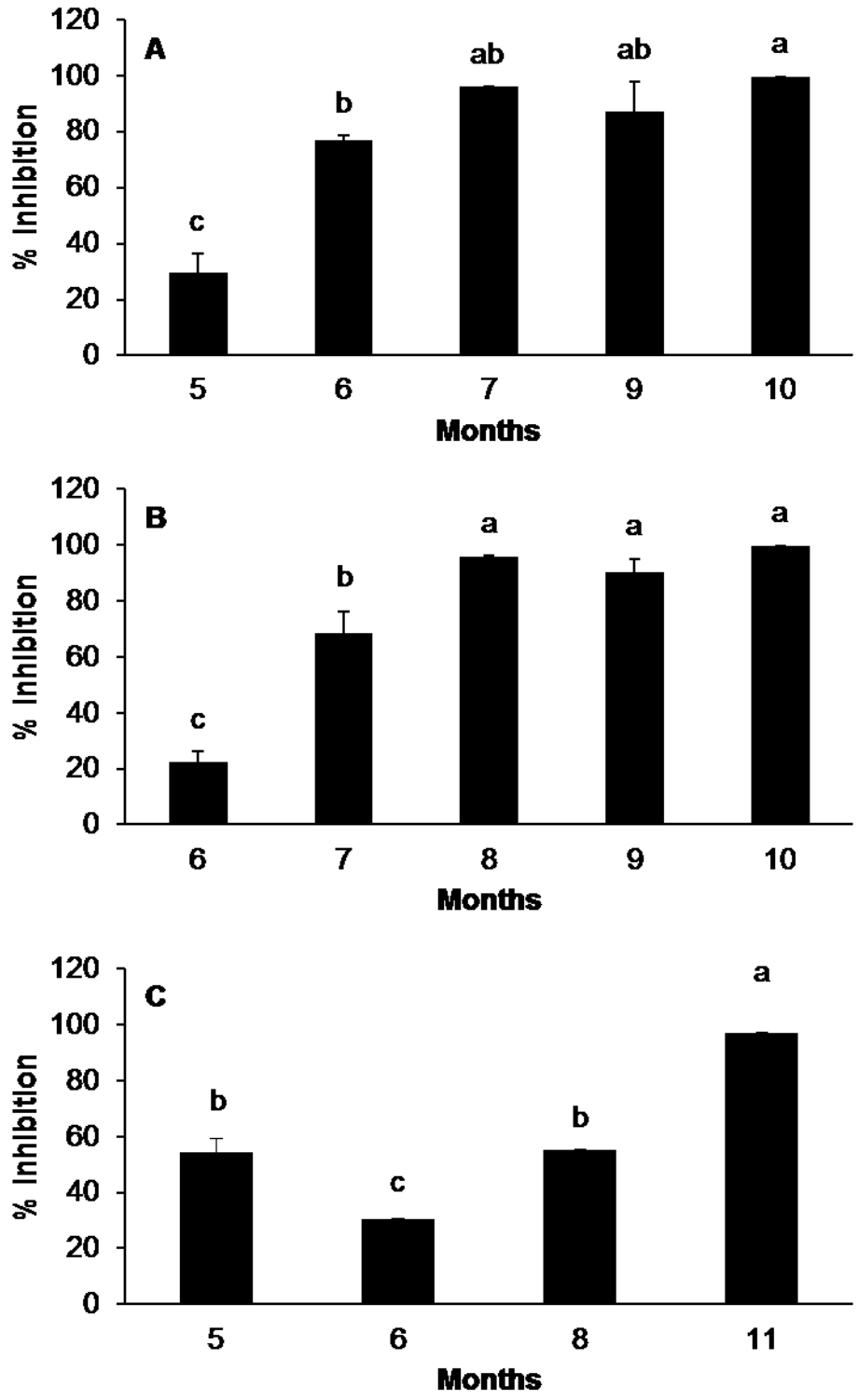

Figure 3-2..α-Glucosidase inhibitory activity induced by fucoidans (0.05 mg/mL) extracted from *A. nodosum* harvested in: A-2002; B-2003 and C-2005. Identical letters within the same panel are not significantly different at $p < 0.05$.

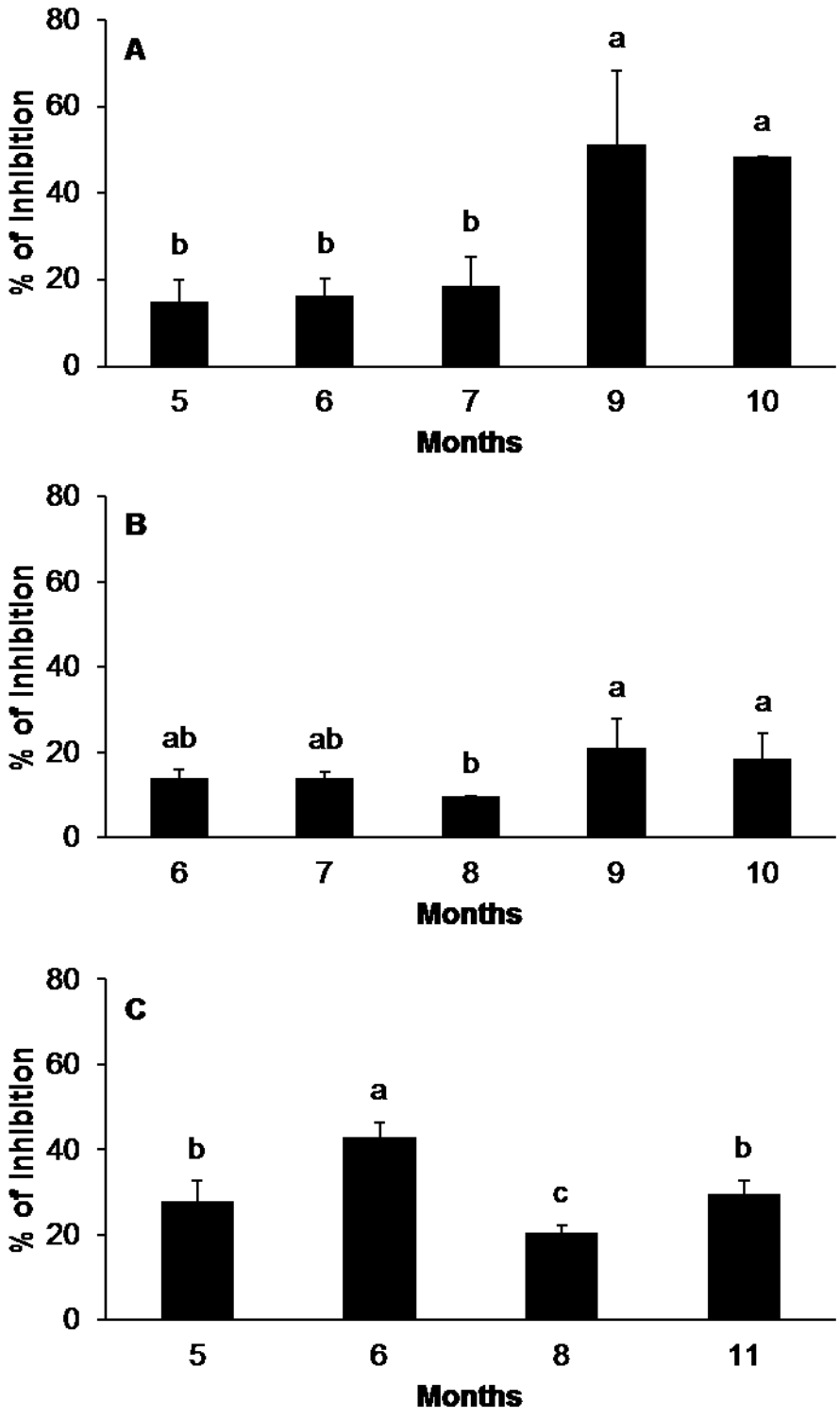

Figure 3-3..α-Glucosidase inhibitory activity induced by fucoidans (0.05 mg/mL) extracted from *F. vesiculosus* harvested in: A-2002; B-2003 and C-2005. Identical letters within the same panel are not significantly different at $p < 0.05$.

Table 3-1. Analysis of variance for the α-amylase measurements

	Degree of freedom	*F*-value	Pr > *F*
Fucoidan concentration: 0.05 mg/mL			
Covariate: Purity	1	0.4	0.5269
Year	2	0.5	0.6326
Months	6	1.3	0.3245
Year*Months	5	0.4	0.8316
Fucoidan concentration: 0.1 mg/mL			
Covariate: Purity	1	0.9	0.3624
Year	2	1.4	0.2909
Months	6	11.3	0.0002
Year*Months	5	7.7	0.0015
Fucoidan concentration: 0.5 mg/mL			
Covariate: Purity	1	0.2	0.6354
Year	2	9.4	0.003
Months	6	15.7	<.0001
Year*Months	5	6.0	0.0044
Fucoidan concentration: 1 mg/mL			
Covariate: Purity	1	0.3	0.5946
Year	2	5.3	0.0208
Months	6	10.8	0.0002
Year*Months	5	2.4	0.0979
Fucoidan concentration: 5 mg/mL			
Covariate: Purity	1	0.3	0.5987
Year	2	29.7	<.0001
Months	6	17.4	<.0001
Year*Months	5	15.6	<.0001

Table 3-2. Variation of the α-amylase inhibitory activity of fucoidans extracted from *A. nodosum* for selected harvests

Harvest month	IC_{50} of fucoidan (mg/ml)*
June	2.301 to 2.515
July	4.189
August	0.124 to 2.699
September	4.520
October	0.471 to 4.621

* Calculation of the fucoidan concentration needed to inhibit 50% of the enzyme activity (realized by Prism software).

Table 3-3. Analysis of variance for the α-glucosidase measurements

		A. nodosum		*F. vesiculosus*	
	Degree of freedom	***F*-value**	**Pr > *F***	***F*-value**	**Pr > *F***
Fucoidan concentration: 0.005 mg/mL					
Covariate: Purity	1	0.19	0.6719	0.03	0.8582
Year	2	0.59	0.5706	1.37	0.2883
Months	6	4.39	0.0122	2.37	0.0901
Year*Months	5	0.27	0.9203	0.92	0.4992
Fucoidan concentration: 0.01 mg/mL					
Covariate: Purity	1	0.08	0.7852	5.44	0.0364
Year	2	1.09	0.3661	1.58	0.2430
Months	6	11.76	0.0001	2.37	0.0906
Year*Months	5	0.72	0.6194	0.97	0.4719
Fucoidan concentration: 0.025 mg/mL					
Covariate: Purity	1	0.27	0.6118	3.88	0.0706
Year	2	5.75	0.0162	6.77	0.0097
Months	6	16.61	<.0001	3.52	0.0270
Year*Months	5	6.53	0.0030	0.72	0.6224
Fucoidan concentration: 0.05 mg/mL					
Covariate: Purity	1	0.00	0.9977	0.21	0.6543
Year	2	8.60	0.0042	13.46	0.0007
Months	6	52.63	<.0001	7.52	0.0012
Year*Months	5	31.90	<.0001	2.61	0.0755

Table 3-4.Variation of the α-glucosidase inhibitory activity of fucoidans extracted from *A. nodosum* for selected harvests

Harvest month	IC_{50} of fucoidan (mg/ml)*
May	0.047
June	0.037
July	0.015 to 0.036
August	0.017 to 0.046
September	0.026 to 0.029
October	0.013
November	0.014

* Calculation of the fucoidan concentration needed to inhibit 50% of the enzyme activity (realized by Prism software).

Table 3-5. Comparision of fucoidans extracted from *A. nodosum* and other food products as inhibitors of α-glucosidase

α-glucosidase inhibitor	IC_{50} (mg/mL)
Fucoidan from *A. nodosum*	0.013~0.047
Green tea*	11.1
Oolong tea*	11.3
Alkaline protease hydrolyzate of sardine muscle*	48.7
Chicken essence*	471.4
Yogurt*	519.8
Acarbose (Type-2 diabetes medication)**	1
Polyphenolic extracts of *A. nodosum* ***	0.024~0.077

*From Matsui et al. (1996) *Biosci. Biotech. Biochem*., 60 (12), 2019-2022
**From Schäfer and Högger (2007) *Diabetes Research and Clinical Practice*, 77, 41-46
*** From Zhang et al. (2007) *Can. J. Physiol. Pharmacol*., 85, 1116-1123.

Chapter 4. Inhibition of alpha-amylase by fucoidan obtained from two species of brown seaweed: the effects of structural characteristics

Kyung-Tae KIM, Laurie-Eve Rioux and Sylvie L. Turgeon

To be submitted to *Phytochemistry*

4.1 Abstract

Fucoidan has shown the possibility of functioning as an inhibitor of α-amylase and α-glucosidase, which is an important discovery in the view of natural health products for diabetes prevention. The inhibitory activity was shown to differ, depending on the seaweed species; the inhibition of α-amylase was only observed with fucoidan from *Ascophyllum nodosum*. To identify the key structural factors of fucoidan that are necessary for its α-amylase inhibitory activity, composition and structural analysis, including glycosidic linkage position, sulfate content and position, and molecular weight, have been comparatively performed using the fucoidans obtained from *Fucus vesiculosus* and *Ascophyllum nodosum*. There was no difference between the two species regarding their monosaccharide composition and type of linkages. The fucoidans obtained from *F. vesiculosus* had lower sulfate content (15.5%) and a higher molecular weight (2351 kDa) as compared to the fraction from *A. nodosum,* 20.6% and 637 kDa, respectively. However, the portion of sulfate groups substituted at C-2/3 and C-4 was similar for both fucoidans. Desulfation of the active fucoidan fractions eliminated its inhibitory activity. The structural characteristics influencing fucoidan's α-amylase inhibitory activity were the molecular weight and the sulfate content. It is suggested that the sulfate groups found in low-molecular fucoidan could easily interact with α-amylase due to their flexible structure. Therefore, both highly sulfated and low-molecular weight fucoidan is suitable to inhibit α-amylase activity.

Keywords: Seaweed, *Fucus vesiculosus, Ascophyllum nodosum*, Fucoidan, α-amylase inhibition, Sulfate group, Monosaccharide, Molecular weight.

4.2 Introduction

Fucoidan is a water-soluble, sulfated polysaccharide and is generally found in brown seaweed such as *Fucus vesiculosus, Ascophyllum nodosum, Ecklonia cava* and *Saccharina longicruris* (Park et al., 2000; Rioux et al., 2007a & b). The major function of fucoidan of seaweed is to prevent its dehydration (Percival and McDowell, 1967). Moreover, therapeutic activities, such as anticancer, anti-HIV, and anticoagulant properties, have been documented. The biological activities of fucoidan were found to be related to its structure (Fujimura et al. 2000).

The structure of fucoidan varies depending on the alga source, the harvesting season and the region (Li et al., 2008; Rioux et al., 2010; Zvyagintseva et al., 2003). Fucoidan from *F. vesiculosus* is mainly composed of α-(1-3) linked sulfated L-fucose (Synytsya et al., 2010; Patankar et al., 1993). In *A. nodosum*, α-(1-3) linked fucose with a low proportion of α-(1-4) linked fucose (Marais and Joseleau, 2001; Daniel et al., 1999) or a repeating α-(1-3) and α-(1-4)-linkage (Chevolot et al., 2001; Daniel et al., 2007) were discovered. Toida et al. (2003) reported that the (1-3)-linkage in fucoidan has a stronger anticoagulation ability than the (1-4)-linkage.

The sulfate content of fucoidan also influences its biological activity, both the anticancer and anticoagulant properties (Toida et al., 2003; Becker et al., 2007; Pereira et al., 2002). Among recent studies, Cho et al. (2011a) reported that oversulfated fucoidan has a better α-amylase inhibitory activity than that of native fucoidan. Others have also shown that the degree of sulfation is an important parameter for its antiviral activity because high amounts of sulfation interferes between the positively charged chain of a viral glycoprotein and the negatively charged hydrogen sulfide at the cell-surface of the glycoprotein receptor (Karmakar et al., 2009). Furthermore, the location of a sulfate group on fucose could affect the biological activity of fucoidan (Tissot et al., 2006; Daniel et al., 2007; Daniel et al., 1999; Anastyuk et al., 2009).

Molecular weight also regulates the biological function of fucoidan; for example, Logeart et al. (1997) showed that high-molecular weight fucoidan inhibited the growth of muscle cells. Also, Yang et al. (2008) reported that partially hydrolyzed fucoidans (~390 - 2200 kDa) were more potent in inducing anticancer activity than native fucoidans (5100 kDa). However, this activity

was decreased at a molecular weight of 260 kDa, possibly because of partial desulfation. The molecular weight of fucoidan varies according to algal species, harvesting period and extraction method. Rioux et al. (2007a) found two different molecular weights for fucoidan in the same seaweed source but using different extraction processes. For example, *A. nodosum* revealed molecular weights of 417 kDa and 1323 kDa, and *F. vesiculosus* revealed molecular weights of 529 kDa and 887 kDa. Patankar et al. (1993) obtained a 100 kDa fucoidan from *F. vesiculosus,* while Rupérez et al. (2002) extracted two fucoidans with molecular weights of 1600 kDa and 43 kDa.

Among the numerous studies on the bioactivity of fucoidan, there has not been a clear elucidation of its potential for diabetes prevention. The inhibition of digestive enzymes by natural compounds has been proposed to delay the increase of blood glucose following starchy food consumption (Kumar et al. 2011). The known natural inhibitors of digestive enzymes include phenolic compounds (Zhang et al. 2007; Spencer et al. 1988; He et al. 2006a and 2006b; Park et al., 1997a; Park et al., 2000; Al-Mamary et al., 2001; Matsui et al. 1996), anthocyanins (Matsui et al. 2001), terpenoids and others. Human salivary α-amylase randomly hydrolyzes α-(1,4)-glucosidic linkages (Bernfeld, 1955). Several amylase inhibitors have been identified, which operate through various mechanisms. Some glucosidic compounds, such as acarbose, which is a pseudo-disaccharide that has a non-reducing end, combine with the enzyme close to the catalytic site and prevent the hydrolysis of starches (Brzozowski and Davies, 1997; Ferey-Roux et al., 1998). Polyphenolic compounds inhibit this enzymatic activity through the precipitation of a polyphenol-enzyme complex (Spencer et al., 1988).

We recently showed the efficiency of fucoidan from the two brown seaweeds *A. nodosum* and *F. vesiculosus* to inhibit α-amylase and α-glucosidase. The fucoidans showed variable inhibitory activity depending on the type of enzyme, for example, α-amylase and α-glucosidase, and the seaweed species from which the fucoidans had been obtained (data shown in Chapter 3). More specifically, the fucoidans obtained from both *A. nodosum* and *F. vesiculosus* inhibited α-glucosidase, but the capacity of inhibition was very different, showing higher activity with *A. nodosum*. Only the fucoidan obtained from *A. nodosum* showed inhibitory activity towards α-amylase. Cho et al. (2011a) also reported a moderate inhibitory effect of fucoidan from

Undaria pinnatifida on α-amyloglucosidase but no effect on α-amylase. The addition of sulfate groups on fucoidan increased the inhibitory effect on α-amylase and α-amyloglucosidase. However, the structural features associated with fucoidan's inhibitory activity remains unknown.

In this study, the structural characterization of fucoidans that were extracted from two brown seaweeds, *Fucus vesiculosus* and *Ascophyllum nodosum*, in Canada was performed to understand the key factors responsible for the α-amylase inhibitory activity. The results were compared to galactofucoidan extracted from *Saccharina longicruris*, whose structure had been previously described (Rioux et al., 2010) and which inhibited α-amylase activity. In addition, we investigated the specificity of α-amylase inhibition by comparing the structure of fucoidan, such as sulfation, monosaccharides composition, glycosidic linkage and molecular weight, between *F. vesiculosus* and *A. nodosum*.

4.3 Materials and methods

4.3.1 Fucoidan

Two types of fucoidans that had been obtained from *Fucus vesiculosus* and *Ascophyllum nodosum* harvested from the St. Laurence River in Quebec in October 2002 were used for this study. The fucoidans were extracted and purified as shown in Chapter 3. Galactofucoidan obtained from *Saccharina longicruris* (Rioux et al., 2010) was utilized as a positive control for fucoidans with an α-amylase inhibitory activity.

4.3.2 Chemical composition

The detection of the protein content of fucoidan samples was performed using a nitrogen analyzer (Leco MI, USA), and 6.25 was used as the conversion factor. The sulfur content was analyzed using ICP-OES (Inductively Coupled Plasma-Optical Emission Spectrometry) with a model Optima 4300DV spectrometer (Perkin Elmer, USA). The following equation was used to determine the sulfate content in fucoidan: Sulfate group (%) = 3.22 × Sulfur % (Roger et al., 2004). The quantification of polyphenol was conducted using spectrometry. Briefly, fucoidan (250 mg) was mixed with 25 mL of acetone-H_2O-HOAc (70:29.5:0.5 (v/v)) and stirred under nitrogen flow. The mixture was sonicated for 20 minutes at room temperature and decanted for 5 minutes. The mixture was filtered using a Whatman #1 filter and rinsed with MeOH. The last two steps were repeated, and then, the filtrate was evaporated until a residual volume of 10 mL was reached. This mixture was stirred in 40 mL of H_2O. A standard curve with gallic acid was prepared, and the Folin-Ciocalteu's phenol reagent (F-9252, Sigma, On, Canada) was used as previously described (Singleton et al., 1999). The absorbance at 765 nm was measured by UV 8543 Agilent spectrophotometer (Agilent, On, Canada).

4.3.3 Molecular weight determination

The molecular weight of the polysaccharide was determined by HPSEC-MALLS (High Performance Size Exclusion Chromatography-Multiangle Laser Light Scattering) (Rioux et al., 2009). A TSK-PWXL guard column (6 mm × 40 mm), TSK-G6000PW (7.5 mm × 300 mm)

and TSK-G4000 PWXL (6 mm × 300 mm) (Tosoh Bioscience, Montgomeryville, USA) columns were used in series. The MALLS system was performed in sequence to the HPSEC procedure. The collected data were evaluated using ASTRA software 4.70.07. The *dn/dc* value applied for fucoidan was 0.129 (Rioux et al., 2007b). As the fucoidans are polydispersed, only the molecular weight averages were compared using the second-order Zimm model.

4.3.4 Monosaccharide analysis

This analysis was performed using a method from Rioux et al. (2009). Briefly, the methyl glycosides in fucoidan were converted to their corresponding per-*O*-trimethylsilylated derivatives by methanolysis. Then, the derivatives were analyzed by gas chromatography (HP 5890A system) with a FID detector. A CP-Sil-5CB fused silica column (60 m × 0.25 mm, Chrompack, Varian) was used. The monosaccharides were identified based upon their retention time and quantified by referencing an internal standard involving myo-inositol. All experiments were conducted in triplicate.

4.3.5 Methylation analysis

Methylation analysis was performed as described in Rioux et al., (2010). The polysaccharide (2 mg) was prepared by lyophilization after being treated with Dowex cation exchange resin (Sigma, On, Canada). Methyl iodide (Sigma, On, Canada) and butyl lithium (Sigma, On, Canada) were used to methylate the hydroxyl groups. Then, the methylated polysaccharide was purified, hydrolyzed, and reduced using sodium borodeuteride (Sigma, On, Canada). The obtained derivatives were acetylated using acetic anhydride (Sigma, On, Canada) and pyridine (Sigma, Canada), analyzed using GC-MS (model 6890N, Agilent Technologies, On, Canada), and identified according to both the retention time and the mass fragment.

4.3.6 Desulfation of fucoidan

Fucoidan desulfation was performed as described in Rioux et al. (2010). The fucoidan solution (10 mg/mL) was purified using Dowex 50WX8-200 ion exchange resin (Sigma, On, Canada).

After the formation of pyridium salt, methanol (4:1 v/v) was added to the sample, and the mixture was heated at 100 ℃ for 4 hours under stirring. The desulfated solution was neutralized and dialyzed using a 1000 Da cut-off membrane (Millipore, USA) over a 48 hour period. Desulfated fucoidan powder was collected after lyophilization.

4.3.7 Structural analysis of sulfate group

Fucoidan samples were analyzed with a Nicolet 506 FT-IR spectrophotometer (Magnar-IR, USA) combined with OMNIC software, version 3.0 (Qiu et al., 2006). KBr pellets were prepared by adding 1 mg of dry fucoidan and 100 mg of KBr. The positions of the sulfate groups were estimated using the method described in Lijour et al. (1994).

4.3.8 Alpha-amylase inhibition assay

The method of Conforti et al. (2005) was modified to determine the inhibitory effects of 1 mg/ml of fucoidan extract on α-amylase (from human salivary, EC 3.2.1.1). The method as shown in Chapter 3 was used.

4.4 Results and Discussion

Fucoidan is a polysaccharide found in brown seaweed. As described Chapter 3, fucoidan from *A. nodosum* efficiently inhibited α-amylase activity (83.2%, Table 4-1), but fucoidan from *F. vesiculosus* did not. Galactofucoidan from *Saccharina longicruris* was utilized as a positive control for α-amylase inhibitory activity (inhibition: 80.3%; Table 4-1) and for structural comparison. To investigate the key factors involved in fucoidan α-amylase inhibitory activity, the structural characterization of the two fucoidan samples obtained from *F. vesiculosus* and *A. nodosum* harvested in October 2002 was performed.

4.4.1 Monosaccharide analysis

The extract was mainly composed of fucoidan (> 80%) and contained residual protein, with an average content of 4% (Chapter 2). The fucoidans from *F. vesiculosus* and *A. nodosum* were mainly composed of fucose, while the galactofucoidan contained a high proportion of galactose (Table 4-2). Glucose and galacturonic acid were not detected in the fucoidan from *A. nodosum,* and only a small amount (< 1%) of these constituents were found in *F. vesiculosus*. The proportion of fucose, galactose, xylose, mannose and glucuronic acid detected in the fucoidan of *F. vesiculosus and A. nodosum* was similar. Also, these results were similar to those reported by Marais and Joseleau (2001) and Nishino et al. (1994). Variations in the monosaccharide composition can be attributed to the different geographical positions and the different extraction methods (Mian and Percival, 1973; Marais and Joseleau, 2001; Percival and McDowell, 1967). When compared with galactofucoidan from *S. longicruris*, both fucoidans contained twice as much fucose and approximately 4-7 fold less galactose. In addition, the ability of fucoidan from *A. nodosum* to inhibit α-amylase was similar to that of galactofucoidan from *S. longicruris.* Considering the difference in the monosaccharide composition among fucoidans and galactofucoidan, the monosaccharide composition is not a factor that influences α-amylase activity.

4.4.2 Glycosidic linkage position

Methylation analysis was performed to determine the monosaccharide linkage position. As shown in Table 4-3, the total composition of the fucosyl residues in fucoidan was 82.91% for *A. nodosum* and 83.51% for *F. vesiculosus*, whereas the galactofucoidan had 41.60% of fucosyl residues. The portion of galactosyl residues was 32.90% in galactofucoidan, 1.20% in *A. nodosum* fucoidan and 8.74% in *F. vesiculosus* fucoidan. The proportion of galactosyl residues in (galacto-) fucoidan was ordered as *S. longicruris* > *F. vesiculosus* > *A. nodosum* (Table 4-3).

According to the results of Rioux et al. (2010), the composition of (1,4)-fucosyl in galactofucoidan of *S. longicruris* was 4.3%. The (1,4)-fucosyl linkage in both fucoidans, 10.17% for *A. nodosum* and 11.82% for *F. vesiculosus*, was higher than that in galactofucoidan. Galactofucoidan showed a slightly higher percentage (11.9%) of the (1,3)-fucosyl linkage than fucoidans (8.19% *A. nodosum* and 7.04% *F. vesiculosus*). In addition, the (1,4)-glucosyl linkage was only detected in *F. vesiculosus* fucoidan (1.47%) but not in *A. nodosum* fucoidan (Table 4-3). The amount of the xyloxyl residue in *A. nodosum, F. vesiculosus* and *S. longicruris* was 13.87%, 6.28% and 5.60%, respectively. Fucoidan from *A. nodosum* contained twice the amount of xylosyl residues than *F. vesiculosus,* but *S. longicruris* contained even less. Overall, the methylation analysis showed few differences between the two fucoidans, but their structures were different from that of galactofucoidan, which suggested that the type of linkage was not the major factor modulating amylase activity.

4.4.3 Sulfate content and their position

The sulfate content was analyzed to investigate its influence on the inhibition of α-amylase (Table 4-4). The total sulfate content of fucoidans was 15.49% for *F. vesiculosus*, 20.64% for *A. nodosum*, and 21.50% for the galactofucoidan of *S. longicruris*. This result shows that the fucoidan of *F. vesiculosus,* which does not exhibit inhibitory activity, has approximately 5% less sulfate groups than the others that do have inhibitory activity, *A. nodosum* and *S. longicruris*.

The location of the sulfate groups on pyranose residues was determined by IR analysis using the method of Lijour et al. (1994). The data obtained were used to indicate whether a specific

position of sulfate groups could regulate the inhibitory activity of α-amylase. According to numerous reports on IR analysis (Qiu et al., 2006; Mähner et al., 2001; Yang et al., 2003; Lijour et al., 1994; Rupérez et al., 2002), sulfate groups found in fucoidan are bound at the equatorial C-2/3 position or at the axial C-4 position. The former position is detected at 840-850 cm^{-1}, whereas the latter is detected at 820 cm^{-1}. As shown in Table 4-4, the proportion of sulfate groups between C-2/3 and C-4 was compared, and there was no difference between the two fucoidans in relation to the location of the sulfate groups. The FT-IR spectrum of the fucoidans (Figure 4.1) showed similar patterns, but variable peak heights were found depending on the source of the fucoidan. This result indicates that the amount of sulfate groups can be variable while the position of sulfate groups in fucoidans is not changed.

The desulfation of fucoidan and galactofucoidan was performed to verify the importance of sulfate groups to α-amylase inhibition. The amount of the remaining sulfate groups found after the desulfation of fucoidan from *A. nodosum* and galactofucoidan from *S. longicruris* was 2.65% and 1%, respectively. The reduction of the sulfate content was shown by FT-IR analysis (Fig. 4-1), in which the peaks heights at 820 cm^{-1}, 848 cm^{-1} and 1259 cm^{-1} were reduced after desulfation of fucoidan from *A. nodosum*. Interestingly, both fucoidan from *A. nodosum* and galactofucoidan from *S. longicruris* completely lost their ability to inhibit α-amylase activity when sulfate groups were removed (Table 4-1). These results clearly indicated the role of sulfate groups as an important factor for fucoidan to control α-amylase activity. However, other structural characteristics are still related to α-amylase inhibition because fucoidan from *F. vesiculosus* did not inhibit α-amylase activity even if it contained 15.49% sulfate groups.

Fucoidan from *A. nodosum* and galactofucoidan possess similar amounts of sulfate groups and α-amylase inhibitory activity. Therefore, the amount of sulfate groups could be an important factor for its bioactivity. Cho et al. (2011a) reported the enhancing effect of the addition of sulfate groups by the chemical oversulfation of fucoidan from *U. pinnatifida* on α-amylase inhibition. In this study, the quantity of reducing sugars produced by α-amylase slightly increased in the presence of native fucoidan, which contained 41.5% sulfate groups, but the

quantity was significantly decreased in presence of oversulfated fucoidan, which contained 51.1% sulfate groups. Because different methods were used to determine the amounts of sulfate groups between studies, it is possible that there is an interaction between the sulfate content and α-amylase inhibition, as seen in the study by Cho et al. (2011a).

Others have demonstrated that sulfate found at the C-2/3 position was required to inhibit factor IIa, (anticoagulant activity) (Toida et al. 2003). Oversulfated fucoidan has suppressed the activity of vascular endothelial growth factor 165 ($VEGF_{165}$) by disturbing the interaction between $VEGF_{165}$ and its receptor on the cell surface, and the inhibitory activity was increased according to the number of sulfate groups found in fucoidan (Koyanagi et al., 2003). According to Tissot et al. (2003), negatively charged fucoidan binds to a part of the positive charges on proteins, for example, antithrombin, through electrostatic interactions. Thus, the electrostatic interaction between the negatively charged sulfate groups of fucoidan and α-amylase might be involved in the modulation of α-amylase activity; however, the exact site of the interaction is not known. Small carbohydrate molecules, such as acarbose, can bind to the active site of α-amylase and hinder the hydrolytic process (Ferey-Roux et al., 1998). An electrostatic interaction between fucoidan and some positively charged amino acids or patches on amylase could modify its conformation and, consequently, its catalytic capability. Sulfated polysaccharides have a well-known affinity for proteins as they are able to interact with proteins at pH levels higher than their isoelectric point (Turgeon et al., 2007). Therefore, sulfate groups found in fucoidan are mandatory for α-amylase inhibition. However, their mechanism of action is still unclear.

4.4.4 Molecular weight analysis

The molecular weights of the fucoidan obtained from *A. nodosum* and galactofucoidan were very similar, 637 kDa and 638 kDa, respectively (Table 4-4). Both polysaccharides also had a similar capacity to inhibit α-amylase activity, 83.2% for fucoidan *A. nodosum* and 80.3% for galactofucoidan (Table 4-1). However, fucoidan from *F. vesiculosus* did not inhibit α-amylase activity, and it had a larger molecular weight (2351 kDa) than the others. Therefore, the molecular weight of the polysaccharides is one of the common characteristics associated with α-amylase inhibition.

The molecular weight of polysaccharides has already been linked to several biological activities. Low-molecular weight fucoidan showed a higher biological activity in several domains, such as anticoagulant (Springer et al., 1957; Zvyagintseva et al., 1999; Nishino and Nagumo, 1992), anticancer (Yang et al., 2008), and anti-HIV (Moen and Clark, 1993; Schaeffer and Krylov, 2000) activities. For example, low-molecular weight fucoidan (approximately 10 to 30 kDa) has stronger anticoagulant activity than native fucoidan (Nishino et al., 1991), and the presence of 2-*O*-sulfation and 2,3-*O*-disulfation are required (Chevolot et al. 1999).

The molecular weight of fucoidan can impact several factors that could influence its efficiency to inhibit α-amylase. First, a higher molecular weight could be associated with a larger conformation that is unfavorable to interact with the enzyme. In contrast, a low-molecular weight could more easily adopt a loose conformation, thus facilitating interactions with the enzyme (Cho et al. 2011b). Secondly, a higher molecular weight results in increased viscosity and, consequently, lower diffusivity in the solvent, thus increasing the time for fucoidan or the substrate to reach the enzyme. Previous work has already shown that fucoidan from *F. vesiculosus* has a higher viscosity than that of *A. nodosum* and *S. longicruris* (Rioux et al., 2007b). Cho et al. (2011a) associated the higher inhibitory activity of the oversulfated fucoidan to its lower molecular weight (165 x 10^3 g/mol) and lower viscosity as compared to native fucoidan (281 x 10^3 g/mol).

From our results, a molecular weight as low as 637 kDa and a sulfate content of 20% for fucoidan were associated with α-amylase activity inhibition. However, there may be other minor constituents that influence the enzymatic activity. Previous work performed on polyphenol and polysaccharides extracted from *A. nodosum* showed antidiabetic activity (Zhang et al., 2007), and the polyphenols were able to inhibit rat intestinal α-glucosidase. To verify if polyphenols were involved in the inhibition of α-amylase, the residual polyphenols of fucoidan extracts were quantified; *A. nodosum* contained 1.00 μg of polyphenol per mg of fucoidan, and *F. vesiculosus* contained 0.98 μg of polyphenol per mg of fucoidan. The residual quantities were low, as the aqueous extraction method was not optimized to retain polyphenolic compounds. Apostolidis and coworker (2010) showed that both α-glucosidase and α-amylase

activity increase with the extraction temperature of polyphenol. At 80 ˚C, an IC_{50} of 0.24 μg and 1.34 μg were found for α-glucosidase and α-amylase, respectively. It is unlikely that the residual polyphenol found in fucoidan had an influence on the inhibition of α-amylase because *F. vesiculosus* would have also been active against α-amylase.

4.5 Conclusion

To identify the structural characteristics of fucoidan involved in the inhibition of α-amylase, comparative investigations have been conducted using different fucoidans from *F. vesiculosus,* which does not exhibit inhibitory activity, and *A. nodosum,* which does exhibit inhibitory activity. Structural similarities were found between both fucoidans, in which the monosaccharide composition, the glycosidic linkage and the position of sulfate groups were similar. Based on the structural analysis, a small molecular weight (637 kDa) and a high sulfate content (21%) are required to inhibit α-amylase activity. Thus, fucoidan might bind to α-amylase throughout electrostatic interactions, changing its conformation in solution and therefore inhibiting its activity. More studies will be needed to verify if a smaller molecular weight (less than 637 kDa) fucoidan would still inhibit α-amylase and to determine the amount of sulfate groups required.

Table 4-1. α-Amylase inhibition of native and desulfated fucoidans (at 1 mg/ml)

	α-amylase inhibition (%)	
Seaweed	Native	Desulfated
F. vesiculosus [a]	0	NA
A. nodosum [a]	83.2 ± 4.0	0
S. longicruris [b]	80.3 ± 0.3	0

[a] Seaweeds were harvested in October 2002.
[b] Rioux et al. (2010) *Phytochemistry* 71, 1586-1595.
NA : not analyzed.

Table 4-2. Monosaccharide composition of fucoidans and galactofucoidan obtained from *A. nodosum*, *F. vesiculosus,* and *S. longicruris*

	A. nodosum Fucoidan[a] (%)	*F. vesiculosus* Fucoidan[a] (%)	*S. longicruris* Galactofucoidan[b] (%)
Fucose	31.1 ± 5.4	24.6 ± 8.4	14.4 ± 1.9
Galactose	4.1 ± 1.7	7.2 ± 1.7	33.1 ± 6.2
Xylose	6.4 ± 2.5	3.9 ± 3.2	2.1 ± 0.2
Mannose	2.9 ± 1.4	2.9 ± 0.8	2.7 ± 0.8
Glucose	0.0 ± 0.0	0.5 ± 0.3	1.4 ± 0.8
Galacturonic acid	0.0 ± 0.0	0.2 ± 0.4	1.0 ± 2.0
Glucuronic acid	2.8 ± 0.8	2.8 ± 0.6	3.2 ± 0.9

[a] The seaweed from which the fucoidan was obtained were harvested in October 2002.
[b] Data were taken from Rioux et al. (2010) *Phytochemistry* 71, 1586-1595.

Table 4-3. Analysis of the methylated, reduced fucoidan alditol acetates.

Glycosyl residues	Position of *O*-methyl groups	Deduced position of residue	Composition (%)		
			A. nodosum Fucoidan[a]	*F. vesiculosus* Fucoidan[a]	*S. longicruris* Galactofucoidan[b]
Fucosyl	2,3,4	*p* Terminal	8.60	10.02	11.40
	2,3,4	*f* Terminal	1.68	1.20	nd
	2,3	4	10.17	11.82	4.30
	2,4	3	8.19	7.04	11.90
	3,4	2	5.59	8.12	nd
	2	3,4	12.59	8.49	3.10
	3	2,4	8.45	8.34	0.50
	4	2,3	12.60	10.79	3.50
		2,3,4	15.04	17.68	6.90
Total			82.91	83.51	41.60
Galactosyl	2,3,4,6	*p* Terminal	nd	2.01	9.00
	2,4,6	3	nd	1.33	6.40
	2,3,4	6	nd	1.51	12.00
	2,4	3,6	1.20	2.30	5.50
	2 or 4	2,4,6 or 2,3,6	nd	1.60	nd
Total			1.20	8.74	32.90
Glucosyl	2,3,6	4	nd	1.47	1.90
Xyloxyl	2,3,4	Terminal	7.75	3.51	5.60
	3,4	2	6.12	2.77	nd
Mannosyl	2 or 4	2,4,6 or 2,3,6	2.02	nd	0.50

[a] The seaweed from which the fucoidan was obtained were harvested in October 2002.

[b] Data were taken from Rioux et al. (2010) *Phytochemistry* 71, 1586-1595.

nd: not detected

Table 4-4. Molecular weight and sulfate group composition and position of fucoidan from different sources.

Analysis / Seaweed	Sulfate content (%)	Position of sulfate group (%)		Molecular weight (kDa)
		C-2/3 [a]	C-4 [b]	
F. vesiculosus	15.49 ± 1.14	46.08	53.92	2351
A. nodosum	20.64 ± 0.33	45.25	54.75	637
S. longicruris [c]	21.50 ± 1.10	NA	NA	638

[a] Detected at 820 cm^{-1}

[b] Detected at 840 cm^{-1}

[c] Data were taken from Rioux et al. (2010) *Phytochemistry* 71, 1586-1595.

NA: not analyzed.

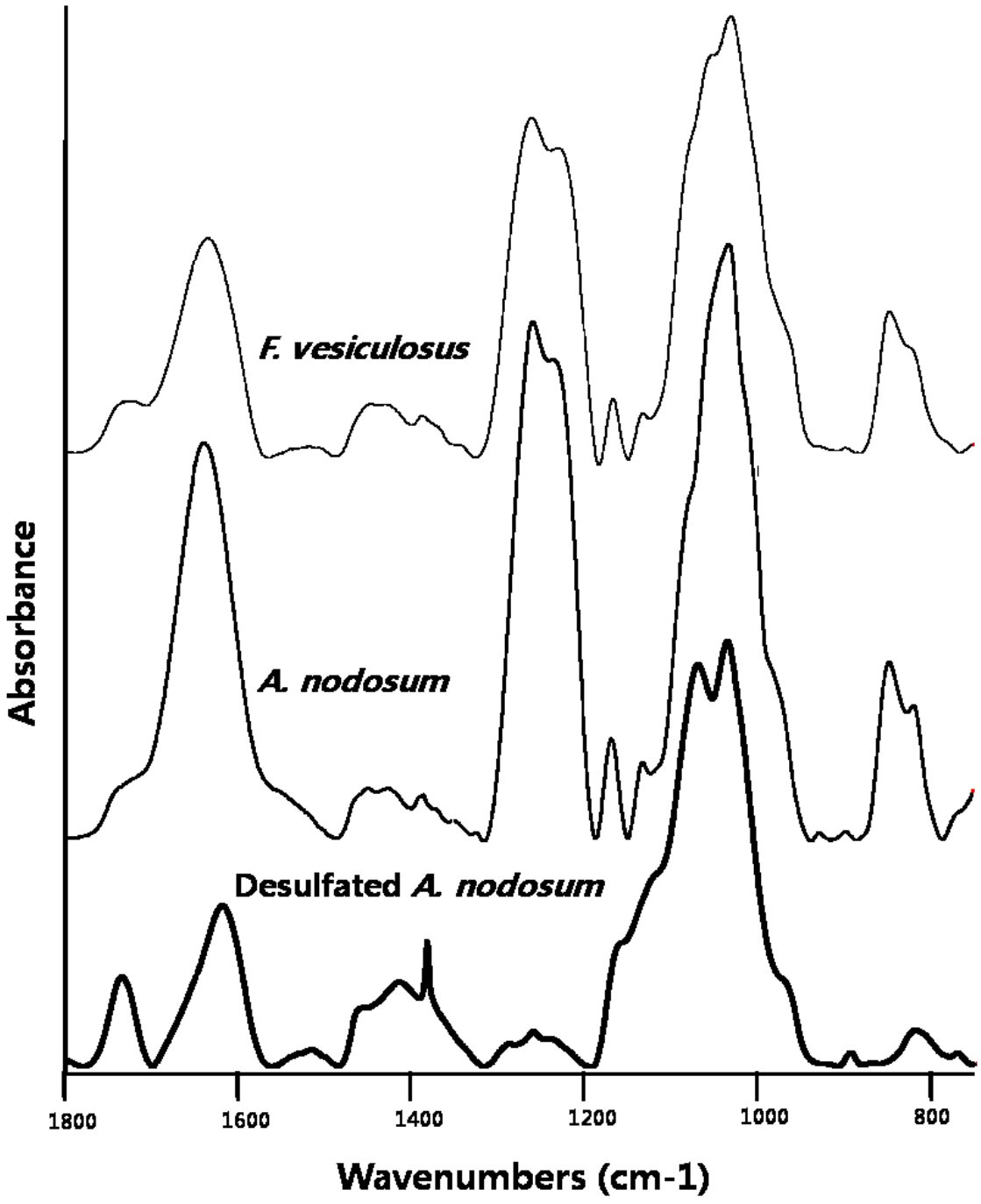

Figure 4-1. FT-IR spectrum of native and desulfated fucoidan.

Chapter 5. General Conclusion and Perspectives

5.1 General Conclusion

This project has been performed to improve the use and the value of brown seaweeds in Quebec through the investigation for harvestable period of seaweed and potential biological function of fucoidan extract with the observation of seasonal influence and the specification of seaweed species.

The hypothesis of this study was that there would be a seasonal variation for chemical components of seaweeds (*F. vesiculosus* and *A. nodosum*) and therefore better condition for seaweed harvest to obtain useful ingredient would exist. The fucoidan which was extracted from the neglected seaweeds in Quebec would have inhibitory ability for starch digestive enzymes (α-amylase and α-glucosidase) and this would improve the value of seaweeds. In addition, understanding of the inhibition mechanism by fucoidan would allow developing functional food for diabetes.

In order to validate this hypothesis, this study has been performed by three objectives;

First, we analyzed the general composition of polysaccharide, protein, lipid, mineral, total phenolic substances and fucoidans of *F. vesiculosus* and *A. nodosum* which were harvested during three years of investigation (2002, 2003 and 2005 year). The seasonal variation depending on seaweed harvesting period and the comparison of ingredient between two species have been estimated. As shown in Chapter 2, the largest content in seaweeds was water to be over 80% (w/w) before lyophilisation. The average composition of polysaccharide, minerals, protein and lipid in dried seaweed was 62.6%, 25.3%, 10.5% and 1.6% for *F. vesiculosus*, and 70.2%, 20.1%, 8.4% and 1.5% for *A. nodosum* respectively. In both seaweeds, the major ingredients were composed of polysaccharide > minerals > protein > lipid > phenols in order (Table 2-1). The average content of other ingredients did not show any remarkable pattern of difference. However, the difference between *F. vesiculosus* and *A. nodosum* was shown depending on harvesting period. From the observation of results, it is concluded that *Fucus vesiculosus* is beneficial for the nutritional base to take more protein (especially from *F. vesiculosus* in May) and mineral (especially from *F. vesiculosus* in summer), (refer to Annex 1 & 3). However, *A. nodosum* contains more polysaccharide than *F. vesiculosus*. For the purpose of obtaining more fucoidan, *Ascophyllum nodosum* in July is advantageous when considering both the yield and its purity (refer to Annex 4 & 5). Therefore, the

choice of either seaweed species or harvesting period can be optimized depending on the purpose of seaweed utilization.

Second, we investigated the fucoidan activity to inhibit starch digestive enzymes (α-amylase and α-glucosidase) depending on seaweed harvesting period and species as shown in Chapter 3. The result of enzyme inhibition test showed that both fucoidans were a potent inhibitor of α-glucosidase by dose-response activity. The fucoidan extracted from *A. nodosum* had higher inhibitory activity for α-glucosidase than the fucoidan of *F. vesiculosus*. The IC_{50} values of *A. nodosum* fucoidan for α-glucosidase inhibition ranged from 0.013 to 0.047 mg/ml, and they were more potent than acarbose (IC_{50} = 1 mg/ml), a well-characterized inhibitor. However, other natural inhibitors like phenolic compounds have shown lower IC_{50} value than acarbose. In the case of α-amylase inhibition, interestingly, *F. vesiculosus* fucoidan did not show the expected activity, whereas the inhibitory activity was detected by *A. nodosum* fucoidan (IC_{50} value: from 0.12 to 4.62 mg/mL). The required fucoidan concentration for α-amylase inhibition (5 mg/ml of fucoidan) was at least 100-fold higher than for α-glucosidase inhibition (0.05 mg/ml of fucoidan). Therefore, fucoidan from *A. nodosum* inhibited both α-amylase and α-glucosidase but the required quantities were much higher for α-amylase inhibition. The optimal period for seaweed harvest to obtain fucoidan which has better activity for α-amylase inhibition was summer/autumn, while the best season to obtain fucoidan for higher α-glucosidase inhibition was autumn. Considering both of fucoidan concentration and the efficiency of glucose reduction, *A. nodosum* harvested in Quebec, has greater potential for the prevention of Type-2 diabetes than *F. vesiculosus*.

Third, we tried to identify the factors which are related to the different α-amylase inhibition activity of fucoidans from *F. vesiculosus* and *A. nodosum*. In order to understand the key structural factors associated with α-amylase inhibitory activity, the characterization of several structural features such as type of linkage, molecular weight, and the content and position of sulfate, were performed by comparing *F. vesiculosus* fucoidan and *A. nodosum* fucoidan with galactofucoidan (extracted from *S. longicruris*) as a positive control for α-amylase inhibition. There was no meaningful difference between *F. vesiculosus* fucoidan and *A. nodosum* fucoidan as shown in the result of monosaccharide composition, linkage and sulfate. More evident differences between the two fucoidans were detected in molecular weight. The fucoidan of *F. vesiculosus* had 2,351 kDa and the fucoidan of *A. nodosum*

had smaller molecular weight of 637 kDa, which was similar to 638 kDa of galactofucoidan having α-amylase inhibitory activity similar to *A. nodosum* fucoidan. In addition to this, it was confirmed that sulfate was related to α-amylase inhibition from the result of desulfation (Table 4-1). For the conclusion, a small molecular weight (637 kDa) and high sulfate content (21%) are required to inhibit α-amylase activity. Thus, fucoidan might bind to α-amylase throughout electrostatic interactions, changing its conformation in solution and therefore inhibiting its activity.

From the results of this study, it is confirmed that brown seaweeds in Quebec have been underestimated and they represent considerable industrial importance for nutritions and bioactive products.

5.2 Perspective

According to the conclusion of this study, the components of seaweed are influenced by harvesting period. This means that various environmental factors such as sunlight, temperature, nitrogen, salinity, tide and phosphate, may contribute to the production of phytochemicals in seaweed. Especially, in the case of seaweeds in Quebec, their habitat is specialized to be extremely severe condition because of long cold period. Therefore, **further estimation for the relationship between environmental factors and seaweed components is required** to decide and to predict the best period to harvest *F. vesiculosus* and *A. nodosum* in Quebec. For more representative pattern to understand how the environmental factors influence seaweed ingredients, long-period monitoring is suggested.

For the functions of fucoidan, in general, there are many cases to be known, such as anti-cancer, anti-coagulation, anti-inflamentation and so on. However, if we consider the seasonal/special variation for the fucoidan activity to inhibit digestive enzymes, it will be useful **to investigate the seasonal/special variation for the other known functions of fucoidan.** We may define better condition of seaweed harvest depending on the targeted function of fucoidan.

Amongst many kinds of seaweed ingredients, we find out the fucoidan having very specific activity to inhibit digestive enzymes of both α-amylase and α-glucosidase. Therefore, it is expected that the fucoidan extracted from *A. nodosum* can be applied for the treatment of Type-2 diabetes and obesity. For the purpose of this application, **proper intake concentration of fucoidan should be estimated to avoid a side-effect like digestive problem** because excess enzyme inhibition can cause abdominal inflation. In addition, there can exist many other useful ingredients in *F. vesiculosus* and *A. nodosum* of Quebec, such as iodine, alginate, polyphenolic compounds (tocopherol, carotenoid) and so on. Potentially, these are good elements for food and pharmaceutic industry, and therefore, we need to **investigate for a novel functional ingredient of seaweed**.

From the conclusion, the mechanism, by which fucoidan obtained from *A. nodosum* inhibits α-amylase (from human saliva), was related to key-factors of low molecular weight and sulfate. However, the extracted fucoidan in the present study has purity less than 90%. It is noted that other minor ingredient can be also involved in the inhibition mechanism. To examine this

possibility, we need **to improve the method of fucoidan extraction. In addition, an improved fucoidan extracting method is needed** to investigate efficiently the binding of enzyme-fucoidan and the inhibition mechanism of fucoidan, In addition, the fucoidan extracting method should be considered to minimize desulfation of fucoidan during molecular weight reducing. To understand α-glucosidase inhibitory mechanism in future study, the relationship between antioxidant capacity and the inhibitory capacity of fucoidan, should be investigated

To know the structural variation of fucoidan depending on season may be helpful to understand detail mechanism. According to Logeart et al. (1997), fucoidan have at least thirty kinds of saccharides to get anti-proliferation of vascular smooth muscle cell. Among them, **how smaller molecular weight of fucoidan** is required for the best inhibition of amylase? **How much of the exposure of sulfate is required** to increase the inhibition? Many questions to complete the mechanism to inhibit α-amylase by the fucoidan obtained from *A. nodosum* can be proposed.

References

Adhikaria, U., Mateub, C.G., Chattopadhyaya, K.C., Pujolb, A., Damonteb, E.B., Ray, B. 2006. Structure and antiviral activity of sulfated fucans from *Stoechospermum marginatum. Phytochemistry*., 67, 2474-2482.

Alam, N., Gourinath, S., Dey, S., Srinivasan, A. and Singh, T.P. 2001. Substrate-inhibitor interactions in the kinetics of a-amylase inhibition by Ragi a-amylase/trypsin inhibitor (RATI) and its various N-terminal fragments. *Biochemistry,* 40, 4229-4233.

Ali H., Houghton P.J., Soumyanath A., 2006. A. α-Amylase inhibitory activity of some Malaysian plants used to treat diabetes; with particular reference to *Phyllanthus amarus*. *Journal of Ethnopharmacology*, 107, 449-455.

Al-Mamary, M., Al-Habori, M., Al-Aghbari, A. and Al-Ob eidi, A. 2001. In vivo effects of dietary sorghum tannins on rabbit digestive enzymes and mineral absorption. *Nutrition Research*, 21, 1393-1401.

Amer, H. Emons, H. and Ostapczuk, P. 1997. Application of multielement techniques for the fingerprinting of elemental contents in *Fucus vesiculosus* from the North Sea. *Chemosphere*, 34, 2123-2131.

Anastyuk, S. D., Shevchenko, N. M., Nazarenko, E. L., Dmitrenok, P. S., and Zvyagintseva, T. N. 2009. Structural analysis of a fucoidan from the brown alga Fucus evanescens by MALDI-TOF and tandem ESI mass spectrometry. *Carbohydrate Research*, 344(6), 779-787.

Anderson, M. Robin, Cardinal, A. and Larochelle, J. 1981. An alternate growth pattern for *Laminaria longicruris. Journal of Phycology,* 17 (4), 405-411

Anno, K., Seno, N. and Ota, M. 1970. Isolation of L-fucose 4-sulfate from fucoidan. *Carbohydrate Research*, 13, 167-169.

AOAC international, 1990, Official methods of analysis of AOAC (Association of official analytical chemists)-15th Edition. Edited by Kenneth Helrich.

Apostolidis, E. and Lee, C.M. 2010. In vitro potential of ascophyllum nodosum phenolic antioxidant-mediated α-glucosidase and α-amylase inhibition. *Journal of Food Science*, 75 (3), H97-H102.

Archer, G.S., Friend, T.H., Caldwell, D., Ameiss, K., Krawczel, P.D., Iacono, C., Keen, H. and Martin, T. 2008. Impacts of feeding several components of the seaweed *Ascophyllum nodosum* on transported lambs. *Animal Feed Science and Technology*, 140, 258-271.

Bacon, L.C. and, R.L. Vadas. 1991 A Model for Gamete Release in *Ascophyllum nodosum (Phaeophyta*). *Journal of Phycology*, 27,166-173

Baron, A. D. 1998. Postprandial hyperglycaemia and alpha-glucosidase inhibitors. *Diabetes Research and Clinical Practice,* 40, S51-S55

Barsanti, L. and Gualtieri, P. 2006. Algae: Anatomy, Biochemistry and Biotechnology. Taylor & Francis Group, *CRC Press*, 320 pages.

Barwell, C.J., Blunden, G. and Manandhar, P.D. 1989. Isolation and characterization of brown algal polyphenols as inhibitors of α-amylase, lipase and trypsin. *Journal of Applied Phycology*, 1 (4), 319-323.

Baum, B.J., 1993. Principles of salivary secretion. *Annals of The New York Academy of Science*. 694, 17–23.

Becker, C. F., Guimaraes, J. A., Mourão, P. A. S. and Verli, H. 2007. Conformation of sulfated galactan and sulfated fucan in aqueous solutions: Implications to their anticoagulant activities. *Journal of Molecular Graphics and Modelling,* 26(1), 391-399.

Bellavia S.L., Moreno J., Sanz E., Picas E.I., Blanco A., 1979. Alpha-amylase activity of human neonate and adult saliva. *Archives of Oral Biology*, 24, 117-121.

Béress, A., Wassermann, O., Tahhan, S., Bruhn, T., Beress, L., Kraiselburd, E.N., 1993. A new procedure for the isolation of anti-HIV compounds (polysaccharide and polyphenols) from the marine algae *Fucus vesiculosus*. *Journal of Natural Products* 56, 478–488.

Bernady G., and Springer, G.F., 1962, Properties of highly purified fucan, *J. Biol. Chem*., 237, 75-80

Bernfeld, P. 1955. Amylases, α and β. *Methods in Enzymology*, 1, 149-158.

Berteau O. and Mulloy B., 2003. Sulfated fucans, fresh perspectives: structures, functions, and biological properties of sulfated fucans and an overview of enzymes active toward this class of polysaccharide. *Glycobiology*, 13(6), 29R-40R.

Bhandari, M.R., Jong-Anurakkun N., Hong G., Kawabata J., 2008. Alpha-glucosidase and alpha-amylase inhibitory activities of Nepalese medicinal herb *Pakhanbhed (Bergenia ciliate, Haw.). Food Chemistry,* 106, 247-252.

Biessels, G.J. and Kappelle, L.J. 2005. Increased risk of Alzheimer's disease in Type II diabetes: insulin resistance of the brain or insulin-induced amyloid pathology? *Biochemical Society Transactions*, 33 (5), 1041-1044

Bilan, M.I, Grachev A.A., Ustuzhanina N.E., 2002, Structure of a fucoidan from the brown seaweed *Fucus evanescens C. Ag. Carbohydrate Research,* 337, 719-730.

Black, W.A.P., 1954. The seasonal variation in the combined L-fucose content of the common British Laminariaceae and fucaceae. *Journal of the Science of Food and Agriculture*, 5, 445-448.

Blumenkrantz, N. and Asboe-Hansen, G., 1973. New method for quantitative determination of uronic acids. *Analytical Biochemistry*, 54, 484-489.

Bobin-Dubigeon, C., Hoebler, C., Longnoné, V., Dagorn-Scaviner, C., Mabeau, S., Barry, J.L. and Lahaye, M. 1997. Chemical composition, physico-chemical properties, enzymatic inhibition and fermentative characteristics of dietary fibres from edible seaweeds. *Sciences des aliments*, 17 (6), 619-639.

Boisson-Vidal, C., Chaubet, F., Chevolot, L., Sinquin, C., Theveniaux, J., Millet, J., Sternberg, C., Mulloy, B. and Fischer, A. M. 2000. Relationship between antithrombotic activities of fucans and their structure. *Drug Development Research*, 51(4), 216-224.

Boney, A.D. 1965. Aspects of the biology of the seaweeds of economic importance. *Advances in Marine Biology*, 3, 105-253.

Borges de Melo, E., Gomes A. and Carvalho, I., 2006. α- and β-Glucosidase inhibitors: Chemical structure and biological activity. *Tetrahedron*, 62, 10277-10302.

Boudet, A.-M. 2007. Review; Evolution and current status of research in phenolic compounds. *Phytochemistry*, 68, 2722–2735

Brand-Miller, J.C., Holt, S.H.A., Pawlak, D.B. and McMillan, J. 2002. Glycemic index and obesity, *The American Journal of Clinical Nutrition,* 76 (1), 281S-285S.

Brasch, D.J., Chang, H.M., Chuah, C.T. and Melton, L.D. 1981. The galactan sulfate from the edible, red alga: *Porphyra columbina*. *Carbohydrate Research,* 91, 113-125.

Braunt, C., Brayer, G.D., Withers, S.G., 1995. Mechanism-based inhibition of yeast α-glucosidase and human pancreatic α-amylase by a new class of inhibitors: 2-DEOXY-2,2-DIFLUORO-α-GLYCOSIDES. *The Journal of Biological Chemistry*, 270, 26778-26781.

Breton, F., Stéphane Cérantola, S. and Gall, E.A. 2011. Distribution and radical scavenging activity of phenols in *Ascophyllum nodosum (Phaeophyceae*). *Journal of Experimental Marine Biology and Ecology,* 399 (2), 167-172.

Brzozowski, A.M. and Davies, G.J. 1997. Structure of the *Aspergillus oryzae* α-amylase complexed with the inhibitor Acarbose at 2.0 Å Resolution. *Biochemistry*, 36, 10837-10845.

Cambridge Dictionaries. Online (http://dictionary.cambridge.org/).

Campo, V.L., Kawano, D.F., da Silva Jr., D.B. and Carvalho, I. 2009. Carrageenans: Biological properties, chemical modifications and structural analysis - A review. *Carbohydrate Polymers*, 77, 167-180.

Canadian Diabetes Association, 2008, 2008 Clinical practice guidelines, *Canadian Journal of Diabetes*, 32 (1), s1-s201.

Chance, R., Baker, A.R., Küpper, F.C., Hughes, C., Kloareg, B. and Malin, G. 2009. Release and transformations of inorganic iodine by marine macroalgae. *Estuarine, Coastal and Shelf Science*, 82, 406-414.

Chapman, A.R.O. and Craigie, J.S. 1978. Seasonal Growth in *Laminaria longicruris*: Relations with reserve carbohydrate Storage and production. *Marine Biology*, 46, 209-213.

Chapman, A.R.O. and Craigie, J.S. 1977. Seasonal growth in *Laminaria longicruris*: Relation with dissolved inorganic nutrients and internal reserves of nitrogen. *Marine Biology*, 40, 197-205.

Chevolot, L., Mulloy, B., Ratiskol, J., Foucault, A., Colliec-Jouault, S. 2001. A disaccharide repeat unit is the major structure in fucoidans from two species of brown algae. *Carbohydrate Research*, 330, 529-535.

Chevolot, L., Foucault, A. Chaubet, F. Kervarec, N., Sinquin, C., Fisher, A.M. and Boisson-Vidal, C. 1999. Further data on the structure of brown seaweed fucans: relationships with anticoagulant activity. *Carbohydrate Research*, 319, 154-165.

Chisti, Y. 2007. Biodiesel from microalgae. *Biotechnology Advances* 25, 294-306.

Cho, M., Han, J.H. and You, S. 2011a. Inhibitory effects of fucan sulfates on enzymatic hydrolysys of starch. *Food Science and Technology*, 44, 1164-1171.

Cho, M.L., Lee, B.Y. and You, S. 2011b. Relationship between oversulfation and conformation of low and high molecular weight fucoidans and evaluation of their in vitro anticancer activity. *Molecules,* 16, 291-297.

Cho, S.Y., H.J. Kang, D.S., Joo, Lee, J.S. and Kim, S.M. 1999, A comparative study on physical properties and gel formation abilities of hot-water extractable material, water-soluble alginate and alkali-soluble alginate extracted from Laminaria japonica in east sea, Korea. *Journal of the Korean Fisheries Society*, 32(6), 774-778.

Choudhary, P. 2004. Review of dietary recommendations for diabetes mellitus. *Diabetes Research and Clinical Practice,* 65S, S9-S15

Church, F. C., Meade, J. B., Treanor, R. E. and Whinna, H. C. 1989. Antithrombin activity of fucoidan - The interaction of fucoidan with heparin cofactor-II, antithrombin-III, and thrombin. *Journal of Biological Chemistry*, 264(6), 3618-3623.

Conchie, J and Percival, E.G.V. 1950. Fucoidin part 2. The hydrolysis of a methylated fucodian prepared from *Fucus vesiculosus, Journal of the Chemical Society*, 827-833.

Conforti, F., Loizzo, M.R., Statti, G.A. and Menichini, F. 2005. Comparative radical scavenging and antidiabetic activities of methanolic extract and fractions from Achillea ligustica All. *Biological & Pharmaceutical Bulletin*, 28 (9), 1971-1794.

Cooper, M.S., Hardin, W.R., Petersen, T.W. and Cattolico, R.A. 2010. Visualizing "green oil" in live algal cells. *Journal of Biocience and Bioengineering,* 109 (2), 198-201.

Coyer, J.A., Hoarau, G., Costa, J.F., Hogerdijk, B., Serrão, E.A., Billard, E., Valero, M. 2011. Evolution and diversification within the intertidal brown macroalgae *Fucus spiralis/F. vesiculosus species* complex in the North Atlantic. *Molecular Phylogenetics and Evolution*, 58 (2), 283-296."

Daniel, R., Chevolot, L., Carrascal, M., Tissot, B., Mourão, P. A. S. and Abian, J. 2007. Electrospray ionization mass spectrometry of oligosaccharides derived from fucoidan of Ascophyllum nodosum. *Carbohydrate Research*, 342(6), 826-834.

Daniel, P.Jr. 2001. Clinical importance of insulin secretion and its interaction with insulin resistance in the treatment of tyep 2 diabetes mellitus and its complications, *Diabetes/Metabolism Research and Reviews*, 17, 181-188.

Daniel, R., Berteau, O., Chevolot, L., Varenne, A., Gareil, P. and Goasdoue, N., 2001. Regioselective desulfation of sulfated L-fucopyranoside by a new sulfoesterase from the marine mollusk Pecten maximus: Application to the structural study of algal fucoidan (*Ascophyllum nodosum*). *European Journal of Biochemistry,* 268, 5617-5626.

Daniel, R., Berteau, O., Jozefonvicz, J. and Goasdoue, N. 1999. Degradation of algal (*Ascophyllum nodosum*) fucoidan by an enzymatic activity contained in digestive glands of the marine mollusk Pecten maximus. *Carbohydrate Research,* 322, 291-297.

Dawczynski, C., Schubert, R. and Jahreis, G. 2007. Amino acids, fatty acids, and dietary fibre in edible seaweed products. *Food Chemistry* 103, 891-899.

Demirbas, A. 2010. Use of algae as biofuel sources. *Energy Conversion and Management*, 51, 2738-2749.

Demirbas, A. and Demirbas, M.F., 2010. Importance of algae oil as a source of biodiesel. *Energy Conversion and Management*, 52(1), 163-170.

Dickinson, C.I. 1963. British Seaweeds. London: Eyre & Spottiswoode,1-65.

Dillon T., Kristensen, K. and O'hEcoha, C. 1953. The seed mucilage of *Ascophyllum nodosum*. *Proceedings of the Royal Irish Academy, Section B: Biological, Geological, and Chemical Science,* 55, 189-194.

Dolečková-Marešová, L., Pavlík, M., Horn, M. and Mareš, M., 2005. De novo design of α-amylase inhibitor: A small linear mimetic of macromolecular proteinaceous ligands. *Chemistry & Biology*, 12, 1349-2357.

Evans, L. V. and Callow,M. E. 1974. Polysaccharide Sulphation in Laminaria, *Planta (Berl.)* 117, 93-95.

Ferey-Roux, G., Perrier, J., Forest, E., Marchis-Mouren, G., Puigserver, A. and Santimone, M. 1998. The human pancreatic α-amylase isoforms: isolation, structural studies and kinetics of inhibition by acarbose. *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology*, 1388 (1), 10-20.

Flexner, C., Barditch-Crovo, P.A., Kornhauser, D.M., Farzadegan, H., Nerhood, L.J., Chaisson, R.E., Bell, K.M. Lorentsen M.J., Hendrix, C.W. and Petty, B.G. 1991. Pharmacokinetics, toxicity, and activity of intravenous dextran sulfate in human immunodeficiency virus infection. *Antimicrobial Agents and Chemotherapy*, 35, 2544-2550.

Fleurence, J. 1999. Seaweed proteins: biochemical nutritional aspects and potential uses. *Food Science & Technology*, 10, 25-28.

Folch, J., Lees, M. and Sloane Stanley, G.H. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *The Journal of Biological Chemistry,* 226, 497-509.

Forsberg, Å, Söderlund, S., Frank, A., Petersson, L.R. and Pedersén, M. 1988. Studies on metal content in the brown seaweed, *Fucus vesiculosus*, from the Archipelago of Stockholm. *Environmental Pollution*, 49, 245-263.

Franco, O.L., Rigden, D.J., Melo, F.R. and Grossi-de-Sa, M. 2002. Plant α-amylase inhibitors and their interaction with insect α-amylases: structure, function and potential for crop protection. *European Journal of Biochemistry*, 269 (2), 397-412.

Franz, M.J. 2000. Protein controversies in diabetes. *Diabetes Spectrum*, 13, 132-141.

Fujimura, T., Shibuya, Y., Moriwaki, S., Tsukahara, K., Kitahara, T., Sano, T., Nishizawa, Y. and Takema, Y., 2000. Fucoidan is the active component of *Fucus vesiculosus* that promotes contraction of fibroblast-populated collagen gels. *Biological & Pharmaceutical Bulletin*, 23 (10), 1180-1184.

Fuse, T. and Goto, F. 1971. Studies on utilization of agar; Part X. Some properties of agarose and agaropectin isolated from various mucilaginous substances of red seaweeds. *Agricultural and Biological Chemistry,* 35 (6), 799-804.

Gagné, J.A., Mann, K.H. and Chapman, A.R.O. 1982. Seasonal patterns of growth and storage in *Laminaria longicruris* in relation to differing patterns of availability of nitrogen in the water. *Marine Biology*, 69, 91-101.

Gamberucci, A., Konta, L., Colucci, A., Giunti, R., Magyar, J.É., Mandl, J., Bánhegyi, G., Benedetti, A., and Csala, M. 2006. Green tea flavonols inhibit glucosidase II. *Biochemical Pharmacology*, 72, 640-646.

Gannon M.C., Nuttall J.A., Damberg G., Gupta V. and Nuttall F.Q., 2001. Effect of protein ingestion on the glucose appearance rate in people with type 2 diabetes. *The Journal of Clinical Endocrinology & Metabolism*., 86, 1040-1047.

Gardiner, G.E., Campbell, A.J., O'Doherty, J.V., Pierce, E., Lynch, P.B., Leonard, F.C., Stanton, C., Ross, R.P. and Lawlor, P.G. 2008. Effect of *Ascophyllum nodosum* extract on growth performance, digestibility, carcass characteristics and selected intestinal microflora poulations of grower-finisher pigs. *Animal Feed Science and Technology*, 141, 259-273.

Geiselaman, J.A. and McConnell, O.J. 1981. Polyphenols in brown algae Fucus vesiculosus and Ascophyllum nodosum: Chemical defenses against the marine herbivorous snail, *Littorina Littorea*. *Journal of Chemical Ecology*, 7 1115-1133.

Geng, P. and Bai, G. 2008. Tow novel amino-oligosaccharides isolated from the culture of *Streptomyces Coelicoflavus ZG0656* as potent inhibitors of α-amylase. *Carbohydrate Research*, 343, 470-476.

Germann, I., Druehl, L.D. and Hoeger, U. 1987. Seasonal variation in total and soluble tissue nitrogen of *Pleurophycus gardneri* (*Phaeophyceae: Laminariales*) in relation to environmentla nitrate. *Marine Biology*, 96, 413-423.

Golay, A., Schneider, H., Temler, E. and Felber, J.P. 1991. Effect of trestatin, an amylase inhibitor, incorporated into bread, on glycemic responses in normal and diabetic patients. *The American Journal of Clinical Nutrition*, 53, 61-65.

Goldberg, R.B., Einhorn, D., Lucas, C.P., Rendell, M.S., Damsbo, P., Huang, W.C., Strange, P. and Brodows, R.G. 1998. A randomized placebo-controlled trial of repaglinide in the treatment of type 2 diabetes. *Diabetes Care*, 21, 1897-1903.

Graham, L. E. and Wilcox, L.W. 2000. Algae, *Upper Saddler River, NJ: Prentice Hall*. 25-29,311-312.

Grant, P. 2010. The perfect diabetes review. *Primary Care Diabetes*, 4, 69-72.

Gupta, S. and Abu-Ghannam, N. 2011. Bioactive potential and possible health effects of edible brown seaweeds. *Trends in Food Science & Technology*, 22, 315-326.

Halvorson, H. and Ellias, L. 1958. The purification and properties of an α-glucosidase of *Saccharomyces Italicus Y1225. Biochimica et Biophysica Acta,* 30, 28-40.

Han, X., Shen, T. and Lou, H. 2007. Dietary polyphenols and their biological significance. *Internal Journal of Molecular Sciences*, 8, 950-988.

Hansawasdi, C., Kawabata, J. and Kasai, T. 2000. α-Amylase inhibitors from Roselle (*Hibiscus sabdariffa Linn*.) tea. *Bioscience, Biotechnology and Biochemistry,* 64 (5), 1041-1043.

Hara, Y. and Honda, M. 1990. The inhibition of α-amylase by tea polyphenols. *Agricultural and Biological Chemistry*, 54 (8), 1939-1945.

Haroun-Bouhedja, F., Ellouali, M., Sinquin, C. and Boisson-Vidal, C. 2000. Relationship between sulfate groups and biological activities of fucans. *Thrombosis Research,* 100 (5), 453-459.

Haug, A. and Larsen, B. 1958. Phenolic compounds in brown algae. I. The presence of reucing compound in *Ascophyllum nodosum (L.) Le Jol. Acta Chemica Scandinavica*, 12, 650-657.

He, Q., Lv Y. and Yao K., 2006a. Effects of tea polyphenols on the activities of alpha-amylase, pepsin, trypsin and lipase. *Food Chemistry*, 101, 1178-1182.

He, Q., Lv Y. and Yao, K. 2006b. Interaction of gallotannins with proteins, amino acids, phospholipids and sugars. *Food Chemistry*, 95, 250-254.

Holdt, S.L. and Kraan, S. 2011. Bioactive compounds in seaweed: functional food applications and legislation. *Journal of Applied Phycology*, 23 (3), 543-597.

Holiman, P.C.H., Hertog, M.G.L. and Katan M.B., 1996. Analysis and health effects of flavonoids. *Food Chemistry*, 57 (1), 43-46.

Hossain, S., Salleh, A., Boyce,A.N., Chowdhury, P.and Naqiuddin, M. 2008. Biodiesel Fuel Production from Algae as Renewable Energy. *American Journal of Biochemistry and Biotechnology*, 4 (3):250-254.

Hou, X.L., Yan, X.J., 1998. Study on the concentration and seasonal variation of inorganic elements in 35 species of marine algae. *Science of the Total Environment*, 222, 141–156.

Ito, K. and Tsuchiya, Y. 1977. Differential fatty acid composition of some marine algae associated with their habitat depths. *Tohoku Journal of Agricultural Research*, 28, 145-150.

Ito, K., and Hori, K. 1989. Seaweed: chemical composition and potential uses. *Food Review International*, 5 (1), 101–144.

Jacobsen, N., Lyche Melvaer, K. and Hensten-Pettersen, A. 1972. Some properties of salivary amylase: A survey of the literature and some observations. *Journal of Dental Research*, 51 (2), 381-388.

Jenkins, D.J.A., 1981. Slow release carbohydrate and the treatment of diabetes. *Proceedings of the Nutrition Society*, 40 (2), 227-235.

Jenkins, D.J.A., Kendall, C.W.C. and Augustin, L.S.A., 2002. Francheschi, S., Hamidi, M. and Marchie, A. Glycemic index: overview of implications in health and disease, *The American Journal of Clinical Nutrition,* 76, 266S-273S

Jiménez-Escrig, A. and Sànchez-Muniz, F.J. 2000. Dietary fibre from edible seaweeds: Chemical structure, physicochemical properties and effects on cholesterol metabolism. *Nutrition Research*, 20 (4), 585-598.

Jiménez-Escrig, A., Jiménez-Jiménez, I., Pulido, R. and Saura-Calixto, F. 2001, Antioxidant activity of fresh and processed edible seaweeds, *Journal of the Science of Food and Agriculture*, 81 (5), 530-534.

Jones, P. and Vogt, T. 2001. Glycosyltransferases in secondary plant metabolism: tranquilizers and stimulant controllers. *Planta*, 213, 164-174.

Jurković, N., Kolb, N. and Colić, I. 1995. Nutritive value of marine algae *Laminaria japonica* and *Undaria pinnatifida*. *Molecular Nutrition & Food Research: Food / Nahrung*, 39 (1), 63-66.

Kahn, S.E. and Porte, D.J. 1997. The pathophysiology of type 2 (noninsulin-dependent) diabetes mellitus: implications for treatment.In: PorteD, Jr, SherwinRS, eds. *Ellenberg & Rifkin's Diabetes Mellitus (5th edition)*., Edited by Stamford, Conn: Appleton & Lange, 487-512.

Kandra, L., Gyemant, G. Zajacz, A. and Batta, G. 2004. Inhibitory effects of tannin on human salivary α-amylase. *Biochemical and Biophysical Research Communications*, 319, 1265-1271.

Kao, Y.H., Chang, H.H. Lee, M.J. and Chen, C.L. 2006. Tea, obesity and diabetes. *Molecular Nutrition & Food Research,* 50 (2), 188-210.

Karmakar, P., Ghosh, T., Sinha, S., Saha, S., Mandal, P., Ghosal, P.K. and Ray, B. 2009. Polysaccharides from the brown seaweed Padina tetrastromatica: Characterization of a sulfated fucan. *Carbohydrate Polymers*, 78(3), 416-421.

Kato, A., Kato, N., Kano, E., Adachi, I., Ikeda, K., Yu, L., Okamoto, T., Banba, Y., Ouchi, H., Takahata, H. and Asano, N., 2005. Biological properties of D- and L-1-deoxyazasugars. *Journal of Medicinal Chemistry*, 48 (6), 2036-2044.

Kim, J.S., Kwon, C.S. and Son, K.H. 2000. Inhibition of alpha-glucosidase and amylase by luteolin, a flavonoid. *Bioscience, Biotechnology and Biochemistry,* 64 (11), 2458-2461.

Kim, K.Y., Nam, K.A., Kurihara, H. and Kim, S.M. 2008. Potent α-glucosidase inhibitors purified from the red alga Grateloupia elliptica. *Phytochemistry*, 69(16), 2820-2825.

Kim, M.J., Lee, S.-B., Lee, H.-B., Lee, S.-Y., Baek, J.-S., Kim, D., Moon, T.-W., Robyt, J.F. and Park, K.-H. 1999. Comparative study of the inhibition of α-glucosidase, α-amylase and

cyclomaltodextrin glucanosyltransferase by acarbose, isoa-carbose, and acarviosine-glucose. *Archives of Biochemistry and Biophysics,* 371 (2), 277-283.

Kim, M.K., Dubacq, J.P., Thomas, J.C. and Giraud, G. 1996. Seasonal variations of triacylglycerols and fatty acids in Fucus serratus. *Phytochemistry*, 43 (1), 49-55.

Kloareg, B., Demarty, M. and Mabeau, S. 1986. Polyanionic characteristics of purified sulphated homofucans from brown algae. *International Journal of Biological Macromolecules,* 8, 380-386.

Kloppenborg, R.P., Van den Berg, E., Kappelle, L.J. and Biessels, G.J. 2008. Diabetes and other vascular risk factors for dementia: Which factor matters most? A systematic review. *European Journal of Pharmacology,* 585, 97-108.

Koo, J.K.1994. Studies on the isolation, purification and characterization of fucoidans from brown algae. *Korea University (Korea). Ph.D thesis.* 123 pages.

Korea Maritime Institute. 1999. Study for demande and consumption pattern of seafood. Basical research report 199-17. P48.

Koyanagi, S., Tanigawa, N., Nakagawa, H., Soeda, S. and Shimeno,H. 2003. Oversulfation of fucoidan enhances its anti-angiogenic and antitumor activities. *Biochemical Pharmacology*, 65, 173-179.

Kumar, S., Narwal, S., Kumar, V. and Prakash, O. 2011. α-glucosidase inhibitors from plants: A natural approach to treat diabetes. *Pharmacognosy Review*. 5(9), 19-29

Kuo, L.C., Polson, A.M. and Kang, T. 2008. Associations between periodontal diseases and systemic diseases: A review of the inter-relationships and interactions with diabetes, respiratory diseases, cardiovascular diseases and osteoporosis. *Public Health*, 122, 417-433.

Küpper, F.C., Schweigert, N., Ar Gall, E., Legendre, J.-M., Vilter, H. and Kloareg, B., 1998. Iodine uptake in Laminariales involves extracellular, haloperoxidase-mediated oxidation of iodide. *Planta*, 207, 163–171.

Kurihara, H., Mitani, T., Kawabata, J. and Takahashi, K. 1999. Two New Bromophenols from the Red Alga Odonthalia corymbifera. *Journal of Natural Products*, 62 (6), 882-884

Kuriki, T. and Imanaka, T. 1999. The concept of the α-amylase family: structural similarity and common catalytic mechanism. *Journal of Bioscience and Bioengineering*, 87(5), 557-565.

Lahaye, M. 1991. Marine algae as sources of dietary fibers: determination of soluble and insoluble dietary fiber contents in some "sea vegetable". *Journal of Science and Food Agriculture*, 54 (4), 587-594.

Lai, E.C.K., Morris, S.A., Street, I.P. and Withers, S.G. 1996. Substituted glycals as probes of glycosidase mechanisms. *Bioorganic & Medicinal Chemistry*, 4 (11), 1929-1937,

Lamela, M., Anca, J., Villar, R., Otero, J. and Calleja, J.M. 1989. Hypoglyucemic acitivity of several seaweed extracts. *Journal of Ethnopharmacology*, 27, 35-43.

Lee, I.K. and Ahn, J.W. 1986. A check list of marine algae in Korea. *The Korean Journal of Phycology*, 1 (1), 311-325

Lee, S.S., Lin, H.C. and Chen, C.K., 2008. Acylated flavonol monorhamnosides, α-glucosidase inhibitors, from Machilus philippinensis. *Phytochemistry*, 69, 2347-2353.

Leeds, A.R. 2002. Glycemic index and heart disease. *The American Journal of Clinical Nutrition,* 6, 286S-289S.

Li, B., Lu, F., Wei, X. and Zhao, W.R. 2008. Fucoidan: structure and bioactivity. *Molecules*, 13, 1671-1695.

Li, N., Zhang, Q. and Song, J. 2005. Toxicological evaluation of fucoidan extracted from Laminaria japonica in Wistar rats. *Food and Chemical Toxicology*, 43, 421–426.

Lijour, Y., Deslandes, G.E. and Guezennec, J. 1994. Estimation of the sulfate content of hydrothermal vent bacterial polysaccharides by fourier transform infrared spectroscopy. *Analytical Biochemistry*, 220, 244-248.

Liu, M., Hansen, P.E. and Lin, X. 2011. Bromophenols in marine algae and their bioactivities. *Marine Drugs,* 9, 1273-1292.

Logeart, D., Prigent-Richard, S., Boisson-Vidal, C., Chaubet, F., Durand, P., Jozefonvicz, J. and Letourneur, D. 1997. Fucans, sulfated polysaccharides extracted from brown seaweed, inhibit vascular smooth muscle cell proliferation. II. Degradation and molecular weight effect. *European Journal of Cell Biology*, 74, 385-390.

Mabeau, S., Kloareg, B. and Joseleau, J. P. 1990. Fractionation and analysis of fucans from brown-algae. *Phytochemistry*, 29(8), 2441-2445.

Mabeau, S. and Fleurence, J. 1993. Seaweed in food products: Biochemical and nutritional aspects. *Trends in Food Science and Technology*, 4, 103-107.

Mähner, C., Lechner, M.D. and Nordmeier, E., 2001. Synthesis and characterisation of dextran and pullulan sulphate. *Carbohydrate Research*, 331, 203-208.

Marais, M.F. and Joseleau, J.P., 2001. A fucoidan fraction from *Ascophyllum nodosum*. *Carbohydrate Research*, 336, 155-159.

Marinho-Soriano, E., Fonseca, P.C., Carneiro, M.A.A. and Moreira, W.S.C. 2006. Seasonal variation in the chemical composition of two tropical seaweeds. *Bioresource Technology*, 97, 2402-2406.

Marks, J.B. and Raskin, P. 2000. Review article; Cardiovascular risk in diabetes: A brief review. *Journal of Diabetes and Its Complication*, 14 (2), 108-115

Mathieson, A.C., Shipman, J.W., O"Shea, J.R. and Hasevlat, R.C. 1976. Seasonal growth and reproduction of estuarine fucoid algae in New England. *Journal of Experimental Marine Biology and Ecology,* 25 (3), 273-284.

Matsui, T., Tanaka, T., Tamura, S., Toshima, A., Tamaya, K., Miyata, Y., Tanaka, K. and Matsumoto, K. 2007. α-Glucosidase inhibitory profile of catechins and theaflavins. *Journal of Agricultural and Food Chemistry*, 55, 99-105

Matsui, T., Ueda, T., Oki, T., Sugita, K., Terahara, N. and Matsumoto, K. 2001. α-Glucosidase inhibitory action of natural acylated anthocyanins. 1. Survey of natural pigments with potent inhibitory activity. *Journal of Agricultural and Food Chemistry*, 49, 1948-1951"

Matsui, T., Yoshimoto, C., Osajima, K., Oki, T. and Osajima, Y. 1996. In vitro survey of α-glucosidase inhibitory food components. *Bioscience, Biotechnology and Biochemistry,* 60 (12), 2019-2022.

Melo, E.B., da Silveira Gomes, A. and Carvalho, I. 2006. α- and β-glucosidase inhibitor: chemical structure and biological activity. *Tetrahedron*, 62, 10277-10302.

Mian, A.J and Percival, E. 1973. Carbohydrates of the brown seaweeds Himanthalia lorea, Bifurcaria bifurcate and Padina pavonia. *Carbohydrate Research*, 26, 133-146.

Miao, H.Q., Ishai-Michaeli, R., Peretz, T. and Voldavsky I. 1995. Laminarin sulfate mimics the effects of heparin on smooth muscle cell proliferation and basic fibroblast growth factor-receptor binding and mitogenic activity. *Journal of Cellular Physiology*, 164 (3), 482-490.

Minami, Y., Kuriyama, C., Ikeda, K., Kato, A., Takebayashi, K., Adachi, I., Fleet, G.W.J., Kettawan, A., Okamoto, T. and Asano, N., 2008. Effect of five-membered sugar mimics on mammalian glycogen-degrading enzymes and various glucosidases. *Bioorganic & Medicinal Chemistry*, 16, 2734-2740.

Mitrakou, A., Kelley, D., Mokan, M., Veneman, T., Pangburn, T., Reilly, J. and Gerich, J., 1992. Role of reduced suppression of glucose production and diminished early insulin release in impaired glucose torerance. *The New England Journal of Medicine,* 326 (1), 22-29.

Moe, S.T., Draget, K.I., Skjåk-Braek, G. and Smidsrød, O. 1995. Alginates (Chapter 9) in "Food polysaccharide and their application". A.M. Stephen (Ed.), Marcel Dekker, New York, 245-286.

Moen L.K., Clark G.F., 1993. A novel reverse transcriptase inhibitor from *Fucus vesiculosus*. *International Conference of AIDS Conference, Jun 6-11; 9: 145 (abstract no. PO-A03-0061).*

Mohan, S. and Pinto, B.M. 2007. Zwitterionic glycosidase inhibitors: Salacinol and related analogues. *Carbohydrate Research*, 342, 1551-1580.

Murray, G. 1947. Anticoagulant therapy with heparin. *The American Journal of Medicine*, 3 (4), 468-471.

Nagaoka, M., Shibata, H., Kimura-Takagi, I., Hashimoto, S., Aiyama, R., Ueyama, S. and Yokokura, T. 2000. Anti-ulcer effects and biological activities of polysaccharides from marine algae, *Biofactors*, 12, 267-274.

Nahoum, V., Roux, G., Anton, V., Rougé, P., Puigserver, A., Bischoff, H., Henrissat, B. and Payan, F. 2000. Crystal structures of human pancreatic α-amylase in complex with carbohydrate and proteinaceous inhibitors. *Biochemical Journal,* 346, 201-208.

Nelson, T.E. and Lewis, B.A. 1974. Separation and characterization of the soluble and insoluble components of insoluble laminaran. *Carbohydrate Research*, 33, 63-74.

Nishino, T. and Nagumo, T. 1992. Anticoagulant and antithrombin activities of oversulfated fucans. *Carbohydrate Research*, 229, 335-362.

Nishino, T., Nagumo, T., Kiyohara, H. and Yamada, H. 1991. Structural characterization of a new anticoagulant fucan sulfate from the brown seaweed Ecklonia kurome. *Carbohydrate Research*, 211 (1), 77-90.

Nishino, T., Nishioka, C., Ura, H. and Nagumo, T. 1994. Isolation and partial characterization of a novel amino sugar-containing fucan sulfate from commercial *Fucus vesiculosus* fucoidan. *Carbohydrate Research,* 255, 213-224.

Norziah, M.H. and Chio, C.Y. 2000. Nutritional composition of edible seaweed Gracilaria changgi. *Food Chemistry*, 68, 69-76.

Nwosu, F., Morris, J., Lund, V.A.., Stewart, D., Ross, H.A. and McDougall, G.J. 2011. Anti-proliferative and potential anti-diabetic effects of phenolic-rich extracts from edible marine algae. *Food Chemistry* 126, 1006–1012.

Obluchinskaya, E.D., Voskoboinikov, G.M. and Galynkin, V.A. 2002. Content of alginic acid and fucoidan in *Fucus* algae of Barents Sea. *Applied Biochemistry and Microbiology*, 38 (2), 186-188.

Ohta, T., Sasaki, S., Oohori, T. Yoshikawa, S. and Kurihara, H. 2002. α-Glucosidase inhibitory activity of a 70% methanol extract from Ezoishige (Pelvetia babingtonii de Toni) and its effect on the elevation of blood glucose level in rats. *Bioscience, Biotechnology and Biochemistry*, 66 (7), 1552-1554.

O'Neil, A.N. 1954. Degradative studies on fucoidan. *Journal of American Chemical Society,* 76 (20), 5074-5076.

Ortega-Calvo, J., Mazuelos, J., Hermosin, C. and Sáiz-Jiménez, C. 1993. Chemical composition of Spirulina and eucaryotic algae food products marketed in Spain. *Journal of Applied Phycology*, 5, 425-435.

Ou, S., Kwok, K.C., Li, Y. and Fu, L. 2001. In vitro study of possible role of dietary fiber in lowering postprandial serum glucose. *Journal of Agricultural and Food Chemistry*, 49, 1026-1029.

Park, H.Y., Cho Y.J., Oh K.S., Koo J.K. and Lee N.G. 2000. Application of seafood processing . *Fishier Publication*, 291-330.

Park, Y.H., Jang D.S. and Kim S.B., 1997a. Utilization of marine products (2nd edition); Chapter 4, Seaweed composition, *Hyoungsul press*, 283-336.

Park, Y.H., Jang D.S. and Kim S.B., 1997b. Utilization of marine products (2nd edition); Chapter 9, Marine product, *Hyoungsul press*, 944-956.

Park,Y.H. 1969. Seasonal variation in the chemical composition of brown algae with special reference to alginic acid. *Bulletin of the Korean Fisheries Society*, 2 (1), 71-82.

Patankar, M.S., Oehninger, S., Barnett, T., Williams, R.L. and Clark, G.F., 1993. A revised structure for fucoidan may explain some of its biological activities. *The Journal of Biological Chemistry*, 268, 21770-21776.

Pedersen, A. 1984. Studies on phenol content and heavy metal uptake in fucoids. *Hydrobiologia*, 116-117, 498-504.

Percival, E.G.V. and Ross, A.G., 1950. Fucoidin. Part I. The isolation and purification of fucoidin from brown seaweeds. *Journal of the Chemical Society*, 717-720.

Percival, E. and McDowell, R.H. 1967. Chemistry and enzymology of marine algal polysaccharides. *Academic Press, London and NEW YORK*, 6-28 & 73-96 &157-174.

Pereira, M. S., Melo, F. R. and Mourão, P. A. S. 2002. Is there a correlation between structure and anticoagulant action of sulfated galactans and sulfated fucans? *Glycobiology,* 12(10), 573-580.

Pereira, M.S., Mulloy, B. and Mourao, P.A.S. 1999. Structure and anticoagulant activity of sulfated fucans: Comparison between the regular, repetitive, and linear fucans from echinoderms with the more heterogeneous and branched polymers from brown algae. *The Journal of Biological Chemistry,* 274 (12), 7656-7667.

Porte, D. Jr. 2001. Clinical importance of insulin secretion and its interaction with insulin resistance in the treatment of type 2 diabetes mellitus and its complications. *Diabetes/Metabolism Research and Reviews*, 17 (3), 181-188.

Prior R. L., Cao, G., Martin, A., Sofic, E., McEwen, J., O'Brien, C., Lischner, N., Ehlenfeldt, M., Kalt, W., Krewer, G. and Mainland, C.M. 1998. Antioxidant capacity as influenced by total phenolic and anthocyanin content, maturity and variety of vaccinium species. *Journal of Agricultural and Food Chemistry*, 46, 2686-2693.

Qian, M., Nahoum, V., Bonicel, J., Bishoff, H., Henrissat, B. and Payan, F. 2001. Enzyme-catalyzed condensation reaction in a mammalian alpha-amylase. High resolution structural analysis of an enzyme inhibitor complex. *Biochemistry*, 40, 7700-7709.

Qiu, X., Amarasekara, A. and Doctor, V. 2006. Effect of oversulfation on the chemical and biological properties of fucoidan. *Carbohydrate Polymers*, 63, 224-228.

Ragan, M.A. and Craigie, J.S. 1976. Physodes and the phenolic compounds of brown algae. Isolation and characterization of phloroglucinol polymers from *Fucus vesiculosus (L.). Canadian Journal of Biochemistry*, 54 (1), 66-73.

Ragan, M.A. and Jensen, A. 1977. Quantitative studies on brown algal phenols. 1. Estimation of absolute polyphenol content of *Ascophyllum nodosum* (L.) Le Jol. and *Fucus vesiculosus* (L.). *Journal of Experimental Marine Biology and Ecology,* 30 (2), 209-221.

Ragan, M.A., Ragan, C.M. and Jensen, A. 1980. Natural chelators in seawater: Detoxification of Zn2+ by brown algal polyphenols. *Journal of Experimental Marine Biology and Ecology,* 44 (2), 261-267.

Ragauskas, A,J., Williams, C.K., Davison, B.H., Britovsek, G., Cairney, J., Eckert, C.A., Frederick, Jr.W.J., Hallett, J.P., Leak, D.J., Liotta, C.L., Mielenz, J.R., Murphy, R., Templer, R. and Tschaplinski, T. 2006. The Path Forward for Biofuels and Biomaterials. *Science*, 311 (5760), 484-489.

Reaven, GM. 1988. Role of insulin resistance in human disease. *Diabetes,* 37, 1595-1607.

Righetti, P.G. and Caravaggio, T. 1976 Isoelectric points and molecular weights of proteins: A table. *Journal of Chromatography*, 1-28.

Rioux, L. E. 2005. Étude de l'influence de la période de récolte de l'algue brune Laminaria longicruris sur sa composition en polysaccharides bioactifs. *Mémoire de maîtrise, Université Laval*.83 pages.

Rioux, L.E., Turgeon, S.L. and Beaulieu, M. 2007a. Characterization of polysaccharides extracted from brown seaweeds. *Carbohydrate Polymers*, 69, 530–537.

Rioux, L.E., Turgeon, S.L and Beaulieu, M. 2007b. Rheological characterisation of polysaccharides extracted from brown seaweeds. *Journal of the Science of Food and Agriculture*, 87 (11), 1630-1638.

Rioux, L.E., Turgeon, S.L. and Beaulieu, M. 2009. Effect of season on the composition of bioactive polysaccharides from the brown seaweed *Saccharina longicruris. Phytochemistry*. 70, 1069-1075.

Rioux, L.E., Turgeon, S.L. and Beaulieu, M. 2010. Structural characterization of laminaran and galactofucan extracted from the brown seaweed *Saccharina longicruris. Phytochemistry*, 71, 1586-1595.

Roehring, K.L. 1988. The physiological effects of dietary fiber. *Food Hydrocolloids*, 2 (1), 1-18.

Roger, O., Kervarec, N., Ratiskol, J. Colliec-Jouault, S. and Chevolot, L. 2004. Structural studies of the main exopolysaccharide produced by the deep-sea bacterium Alteromonas infernus. *Carbohydrate Research*, 339, 2371-2380.

Roth, J., Ziak M. and Zuber C. 2003. The role of glucosidase II and endomannosidase in glucose trimming of asparagine-linked oligosaccharides. *Biochimie*, 85, 287-294.

Round, F.E. 1981. The ecology of algae. *Cambridge University Press*, 63-77 and 421-483.

Rupérez, P. 2002. Mineral content of edible marine seaweeds. *Food Chemistry*, 79, 23-26.

Rupérez, P., Ahrazem, O. and Leal, J. A. 2002. Potential antioxidant capacity of sulphated polysaccharides from edible brown seaweed Fucus vesiculosus. *Journal Agricultural Food Chemistry*, 50, 840–845.

Sánchez-Machado, D.I., López-Cervantes, J., López-Hernández, J. and Paseiro-Losad, P. 2004. Fatty acids, total lipid, protein and ash contents of processed edible seaweeds. *Food Chemistry*, 85, 439-444.

Schaeffer, D.J. and Krylov, V.S. 2000. Anti-HIV activity of extracts and compounds from algae and cyanobacteria. *Ecotoxicology and Environmental Safety*, 45, 208-227.

Schäfer, A. and Högger, P. 2007. Oligomeric procyanidins of French maritime pine bark extract (Pycnogenol) effectively inhibit a-glucosidase. *Diabetes Research and Clinical Practice*, 77, 41-46.

Schweiger R.G., 1962. Methanolysis of Fucoidan. II. The Presence of Sugars Other Than L-Fucose. *The Journal of Organic Chemistry*, 27 (12),4270–4272

Seo, W.D., Kim, J.H., Kang, J.E., Ryu, H.W., Curtis-Long, M.J., Lee, H.S., Yang, M.S. and Park, K.H., 2005. Sulfonamide chalcone as a new class of α-glucosidase inhibitors. *Bioorganic & Medicinal Chemistry Letters*, 15, 5514-5516.

Sheader, A. and Moss, B. 1975. Effect of light and tempereature on germination and growth of *Ascophyllum nodosum* (L.) Le Jol. *Estuarine and Coastal Marine Science*, 3, 125-132.

Shinde, J., Taldone, T., Barletta, M., Kunaparaju, N., Hu, B., Kumar, S., Placido, J. and Zito, W., 2008. a-Glucosidase inhibitory activity of Syzygium cumini (Linn.) Skeels seed kernel in vitro and in Goto–Kakizaki (GK) rats. *Carbohydrate Research*, 343, 1278–1281

Sigma-Aldrich, 2007. Complex Carbohydrate Analysis: Enzymes, Kits, and Reagents. *Biofiles for life science research,* 2, 1-24.

Singleton, V. L., Orthofer, R., and Lamuela-Raventós, R. M. 1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. *In Methods in Enzymology : Academic Press,* 152-178.

Sinnott, M.L. 1990. Catalytic Mechanisms of Enzymic Glycosyl Transfer. *Chemical Reviews*, 90 (7). 1171-1202.

Skriptsova, A. V., Shevchenko, N. M., Zvyagintseva, T. N. and Imbs, T. I. 2010. Monthly changes in the content and monosaccharide composition of fucoidan from Undaria pinnatifida (Laminariales, Phaeophyta). *Journal of Applied Phycology*, 22(1), 79-86.

Slinkard, K. and Singleton, V.L. 1997. Total phenol analysis : Automation and comparison with manual methods. *American Journal of Enology and Viticulture*, 28, 49-55.

So, M.J., Kim, B.K., Choi,M.J., Park, K.Y.,Rhee, S.H. and Cho, E.J. 2007. Protective activity of fucoidan and alginic acid against free radical-induced oxidative stress under in vitro and cellular system. *Journal of Food Science and Nutrition*, 12 (4), 191-196.

Soeda, S., Kozako, T., Iwata, K. and Shimeno, H. 2000. Oversulfated fucoidan inhibits the basic fibroblast growth factor-induced tube formation by human umbilical vein endothelial cells: its possible mechanism of action. *Biochimica et Biophysica Acta*., 1497, 127-134.

Søgaard, M., Abe, J., Martin-Eauclaire, M.F. and Svensson, B. 1993. α-Amylase: Structure and function. *Carbohydrate Polymers*, 21, 137-146.

Song, Y., Manson, J.E., Buring, J.E., Sesso, H.D. and Liu, S., 2005. Associations of dietary flavonoids with risk of type 2 diabetes, and markers of insulin resistance and systemic inflammation in women: a prospective study and cross-sectional analysis. *Journal of the American College of Nutrition*, 24 (5), 376-384.

Souchet, N. 2004. Étude de l'influence de la période de récolte de l'algue brune *Laminaria longicruris* sur sa composition en polysaccharides bioactifs. *Mémoire de maîtrise, Université Laval*.82 pages.

Spencer, C.M., Cai, Y., Martin, R., Gaffney, S.H. Goulding, P.N., Magnolato, D., Lilley, T.H. and Haslam, E. 1988. Polyphenol complexation-some thoughts and observations. *Phytochemistry*, 27 (8), 2397-2409.

Springer, G. F., Wurzel, H. A. and Mcsneal, G. M., 1957. Isolation of anticoagulant fractions from crude fucoidan. *Proceedings of the Society for Experimental Biology and Medicine*, 94, 404–409.

Stewart, C. M., Higgins, H. G. and Austin, S., 1961. Seasonal variation in alginic acid, mannitol, laminarin and fucoidan in the brown alga, *Ecklonia radiata*. *Nature*, 192, 1208.

Stewart, W.D.P. 1974. Algal physiology and biochemistry. University of California Press. 561-675.

Stringer, S. E. and Gallagher, J.T. 1997. Heparan sulphate. *The International Journal of Biochemistry & Cell Biology*, 29 (5), 709-714.

Strobl, S.,Muehlhahn, P., Bernstein, R.,Wiltscheck, R., Maskos, K.,Wunderlich, M., Huber, R., Glockshuber, R. and Holak, T.A. 1995. Determination of the three dimensional structure of the bifunctional a-amylase/trypsin inhibitors from Ragi seeds by NMR spectroscopy. *Biochemistry*, 34, 8281-8293.

Strömgren, T. 1986. Annual variation in growth rate of perennial littoral fucoid algae from the west coast of Norway. *Aquatic Btany*, 23, 361-369

Svensson, B., Fukuda, K., Nielsen, P.K. and Bønsager, B.C. 2004. Proteinaceous α-amylase inhibitors. *Biochimica et Biophysica Acta*., 1696, 145-156.

Synytsya, A., Kim, W.J., Kim, S.M., Pohl, R., Synytsya, A., Kvasnicka, F., Copikova, J. and Park, Y.I., 2010. Structure and antitumor activity of fucoidan isolated from sporophyll of Korean brown seaweed *Undaria pinnatifida. Carbohydrate Polymers*, 81, 41-48.

Teeri, T.T., 1991. Engineering of enzymes of carbohydrate metabolism. *Current Opinion in Biotechnology*, 2, 614-621.

Tewari, N., Tiwari, V.K., Mishra, R.C., Tripathi, R.P., Srivastava, A.K., Ahmad, R., Srivastava, R., Srivastava, B.S., 2003. Synthesis and bioevaluation glycosyl ureas as alpha-glucosidase inhibitors and their effect on mycobacterium. *Bioorganic & Medicinal Chemistry*, 11 (13), 2911-2922.

Thalapaneni, N.R., Chidambaram, K.A., Ellappan, T., Sabapathi, M.L. and Mandal, S.C. 2008. Inhibition of carbohydrate digestive enzymes by Talinum portulacifolium (Forssk) leaf extract. *Journal of Complementary and Integrative Medicine,* 5 (1), Article 11. 1-10.

Tilden J.E., 1935, The Algae and their life relations. Fundamentals of Phycology. Second edition. Minneapolis: *University of Minnesota Press*. 550 pages

Tissot, B., Salpin, J. Y., Martinez, M., Gaigeota, M. P. and Daniel, R. 2006. Differentiation of the fucoidan sulfated L-fucose isomers constituents by CE-ESIMS and molecular modeling. *Carbohydrate Research*, 341(5), 598-609.

Tissot, B., Montdargent, B., Chevolot, L., Varenne, A., Descroix, S., Gareil, P. and Daniel, R., 2003. Interaction of fucoidan with the proteins of the complement classical pathway. *Biochimica et Biophysica Acta,* 1651, 5-16.

Toida , T., Chaidedgumjorn, A. and Linhardt, R.J. 2003. MINIREVIEW-Structure and Bioactivity of sulfated polysaccharides. *Trends in Glycoscience and Glycotechnology*, 15 (81), 29-46.

Turgeon, S.L., Schmitt, C. and Sanchez, C. 2007. Protein-polysaccharide complexes and coacervates. *Current Opinion in Colloid & Interface Science*, 12, 166-178.

UK Prospective Diabetes Study (UKPDS) Group, 1998. Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). *Lancet*, 352, 837-853.

Usov, A.I., Smirnova, G.P. and Klochkova, N.G. 2001. Polysaccharide of algae: 55. Polysaccharide composition of several brown algae from Kamchatka. *Russian Journal of Bioorganic chemistry*, 27(6), 395-399.

van Netten, C., Cann, S.A.H., Morley, D.R., van Netten, J.P., 2000. Elemental and radioactive analysis of commercially available seaweed. *Science of the Total Environment*, 255, 169–175.

Wang, F. 1998. Focus on repaglinide: an oral hypoglycemic agent with a more rapid onset and shorter duration of action than the sulfonylureas. *Formulary,* 33, 409-423.

Wang, J., Li, L., Zhang, Q., Zhang, Z., Qi, H. and Li, P. 2009b. Synthesized oversulphated, acetylated and benzoylated derivatives of fucoidan extractedd from *Laminaria japonica* and their potential antioxidant activity in vitro. *Food Chemistry*, 114, 1285-1290.

Wang, T., Jónsdóttir, R. and Ólafsdóttir, G. 2009a. Total phenolic compounds, radical scavenging and metal chelation of extracts from Icelandic seaweeds. *Food Chemistry*, 116, 240–248.

Wang, Y., Xu, Z., Bach, S.J. and McAllister, T.A. 2008. Effects of phlorotannins from *Ascophyllum nodosum* (brown seaweed) on in vitro ruminal digestion of mixed forage or barley grain. *Animal Feed Science and Technology*, 145, 375-395.

Weng, J.K., Li, X., Bonawitz, N.D. and Chapple, C. 2008. Emerging strategies of lignin engineering and degradation for cellulosic biofuel production. *Current Opinion in Biotechnology*, 19,166–172.

Wi, S.G., Kim, H.J., Mahadevan, S.A., Yang, D.J. and Bae, H.J. 2009. The potential value of the seaweed Ceylon moss (Gelidium amansii) as an alternative bioenergy resource. *Bioresource Technology*, 100, 6658-6660.

Wild, S., Roglic, G., Green, A., Sicree, R. and King, H. 2004. Global prevalence of diabetes estimates for the year 2000 and projections for 2030. *Diabetes Care*, 27, 1047-1053.

Wolever, T.M.S., Katzman-Relle, L., Jenkins, A.L., Vuksan, V, Josse, R.G. and Jenkins, D.J.A., 1994. Glycaemic index of 102 complex carbohydrate foods in patients with diabetes. *Nutrition Research*, 14, 651-669.

Yaich, H., Garna, H., Besbes, S., Paquot, M., Blecker, C. and Attia, H. 2011. Chemical composition and functional properties of *Ulva lactuca* seaweed collected in Tunisia. *Food Chemistry*, 128 (4), 895-901.

Yang, C., Chung, D., Shin, I., Lee, H. Kim, J., Lee, Y. and You, S. 2008. Effects of molecular weight and hydrolysis conditions on anticancer activity of fucidans from Sporophyll of Undaria pinnatifida. *International journal of Biological Macromolecules*, 43, 433-437.

Yang, J., Du, Y., Wen, Y., Li, T. and Hu, L. 2003. Sulfation of chinese lacquer polysaccharides in different solvents. *Carbohydrate Polymers*, 52, 397-403.

Yu, S., Bojsen, K., Svensson, B. and Marcussen, J. 1999. α-1,4-Glucan lyases producing 1,5-anhydro-D-fructose from starch and glycogen have sequence similarity to α-glucosidases. *Biochimica et Biophysica Acta*, 1433, 1-15.

Zemani, F., Benisvy, D., Galy-Fauroux, I., Lokajczyk, A., Colliec-Jouault, S., Uzan, G. and Fischer, A.M. 2005. Low-molecular-weight fucoidan enhances the proangiogenic phenotype of endothelial progenitor cells. *Biochemical Pharmacology*, 70, 1167-1175

Zhang J., Tiller C., Shen J., Wnag C., Girouard G., Dennis D., Barrow C., Miao M., Ewart S., 2007. Antidiabetic properties of polysaccharide- and polyphenolic-enriched fractions from the brown seaweed Ascophyllum nodosum. *Canadian Journal of Physiology and Pharmacology,* 85 (11), 1116-1123.

Zvyagintseva, T.N., Shevchenko, N.M., Chizhov, A.O., Krupnova, T.N., Sundukova, E.V., and Isakov, V.V. 2003. Water-soluble polysaccharides of some far-eastern brown seaweeds. Distribution, structure, and their dependence on the developmental conditions. *Journal of Experimental Marine Biology and Ecology*, 294 (1), 1-13.

Zvyagintseva, T.N., Shevchenko, N.M., Popivnich, I.B., Isakov, V.V., Scobun, A.S., Sundukova, E.V., and Elyakova, L.A., 1999. A new procedure for the separation of water-soluble polysaccharides from brown seaweeds. *Carbohydrate Research*, 322, 32-39.

ANNEX

Summary	Wording	P - value
***	Extremely significant	< 0.001
**	Very significant	0.001 to 0.01
*	Significant	0.01 to 0.05
ns	Not significant	> 0.05

Annex 1. Two-way analysis of Protein content of *F. vesiculosus* and *A. nodosum* (Month*Seaweed)

Two-way ANOVA				
Source of Variation	% of total variation	P value		
Interaction	4.86	0.0007		
Seaweed	32.88	< 0.0001		
Month	46.01	< 0.0001		
Source of Variation	P value summary	Significant?		
Interaction	***	Yes		
Seaweed	***	Yes		
Month	***	Yes		
Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	5	11.35	2.269	4.895
Seaweed	1	76.81	76.81	165.7
Month	5	107.5	21.50	46.37
Residual	71	32.91	0.4636	
Number of missing values	25			
Bonferroni posttests				
Fucus vs Asco				
Month	Fucus	Asco	Difference	95% CI of diff.
May	13.22	10.76	-2.464	-3.531 to -1.397
June	11.08	8.554	-2.526	-3.397 to -1.655
July	9.155	8.230	-0.9246	-1.992 to 0.1423
Aug	9.095	8.251	-0.8442	-1.911 to 0.2227
Sep	9.937	7.144	-2.793	-3.913 to -1.674
Oct&Nov	10.08	7.839	-2.242	-3.113 to -1.371
Month	Difference	t	P value	Summary
May	-2.464	6.269	P<0.001	***
June	-2.526	7.871	P<0.001	***
July	-0.9246	2.352	P > 0.05	ns
Aug	-0.8442	2.148	P > 0.05	ns
Sep	-2.793	6.776	P<0.001	***
Oct&Nov	-2.242	6.985	P<0.001	***

Bonferroni posttests				
May vs June				
Seaweed species	May	June	Difference	95% CI of diff.
Fucus	13.22	11.08	-2.144	-3.317 to -0.9711
Asco	10.76	8.554	-2.206	-3.379 to -1.033
Seaweed species	Difference	t	P value	Summary
Fucus	-2.144	5.975	P<0.001	***
Asco	-2.206	6.147	P<0.001	***
May vs July				
Seaweed species	May	July	Difference	95% CI of diff.
Fucus	13.22	9.155	-4.070	-5.355 to -2.785
Asco	10.76	8.230	-2.530	-3.815 to -1.245
Seaweed species	Difference	t	P value	Summary
Fucus	-4.070	10.35	P<0.001	***
Asco	-2.530	6.436	P<0.001	***
May vs Aug				
Seaweed species	May	Aug	Difference	95% CI of diff.
Fucus	13.22	9.095	-4.130	-5.415 to -2.845
Asco	10.76	8.251	-2.510	-3.795 to -1.225
Seaweed species	Difference	t	P value	Summary
Fucus	-4.130	10.51	P<0.001	***
Asco	-2.510	6.384	P<0.001	***
May vs Sep				
Seaweed species	May	Sep	Difference	95% CI of diff.
Fucus	13.22	9.937	-3.288	-4.573 to -2.003
Asco	10.76	7.144	-3.617	-4.964 to -2.269
Seaweed species	Difference	t	P value	Summary
Fucus	-3.288	8.363	P<0.001	***
Asco	-3.617	8.772	P<0.001	***
May vs Oct&Nov				
Seaweed species	May	Oct&Nov	Difference	95% CI of diff.
Fucus	13.22	10.08	-3.144	-4.317 to -1.971
Asco	10.76	7.839	-2.921	-4.094 to -1.748
Seaweed species	Difference	t	P value	Summary
Fucus	-3.144	8.761	P<0.001	***
Asco	-2.921	8.141	P<0.001	***
June vs July				
Seaweed species	June	July	Difference	95% CI of diff.
Fucus	11.08	9.155	-1.926	-3.099 to -0.7526
Asco	8.554	8.230	-0.3240	-1.497 to 0.8490
Seaweed species	Difference	t	P value	Summary
Fucus	-1.926	5.366	P<0.001	***
Asco	-0.3240	0.9028	P > 0.05	ns
June vs Aug				
Seaweed species	June	Aug	Difference	95% CI of diff.
Fucus	11.08	9.095	-1.986	-3.159 to -0.8127
Asco	8.554	8.251	-0.3037	-1.477 to 0.8693
Seaweed species	Difference	t	P value	Summary
Fucus	-1.986	5.534	P<0.001	***
Asco	-0.3037	0.8463	P > 0.05	ns
June vs Sep				
Seaweed species	June	Sep	Difference	95% CI of diff.
Fucus	11.08	9.937	-1.144	-2.317 to 0.02948

Asco	8.554	7.144	-1.411	-2.652 to -0.1694
Seaweed species	Difference	t	P value	Summary
Fucus	-1.144	3.187	P<0.01	**
Asco	-1.411	3.715	P<0.001	***
June vs Oct&Nov				
Seaweed species	June	Oct&Nov	Difference	95% CI of diff.
Fucus	11.08	10.08	-0.9997	-2.049 to 0.04951
Asco	8.554	7.839	-0.7153	-1.764 to 0.3339
Seaweed species	Difference	t	P value	Summary
Fucus	-0.9997	3.115	P<0.01	**
Asco	-0.7153	2.229	P > 0.05	ns
July vs Aug				
Seaweed species	July	Aug	Difference	95% CI of diff.
Fucus	9.155	9.095	-0.06013	-1.345 to 1.225
Asco	8.230	8.251	0.02027	-1.265 to 1.305
Seaweed species	Difference	t	P value	Summary
Fucus	-0.06013	0.1530	P > 0.05	ns
Asco	0.02027	0.05157	P > 0.05	ns
July vs Sep				
Seaweed species	July	Sep	Difference	95% CI of diff.
Fucus	9.155	9.937	0.7821	-0.5029 to 2.067
Asco	8.230	7.144	-1.087	-2.434 to 0.2609
Seaweed species	Difference	t	P value	Summary
Fucus	0.7821	1.989	P > 0.05	ns
Asco	-1.087	2.636	P < 0.05	*
July vs Oct&Nov				
Seaweed species	July	Oct&Nov	Difference	95% CI of diff.
Fucus	9.155	10.08	0.9259	-0.2471 to 2.099
Asco	8.230	7.839	-0.3913	-1.564 to 0.7817
Seaweed species	Difference	t	P value	Summary
Fucus	0.9259	2.580	P < 0.05	*
Asco	-0.3913	1.090	P > 0.05	ns
Aug vs Sep				
Seaweed species	Aug	Sep	Difference	95% CI of diff.
Fucus	9.095	9.937	0.8422	-0.4428 to 2.127
Asco	8.251	7.144	-1.107	-2.455 to 0.2406
Seaweed species	Difference	t	P value	Summary
Fucus	0.8422	2.142	P > 0.05	ns
Asco	-1.107	2.685	P < 0.05	*
Aug vs Oct&Nov				
Seaweed species	Aug	Oct&Nov	Difference	95% CI of diff.
Fucus	9.095	10.08	0.9861	-0.1870 to 2.159
Asco	8.251	7.839	-0.4116	-1.585 to 0.7614
Seaweed species	Difference	t	P value	Summary
Fucus	0.9861	2.748	P < 0.05	*
Asco	-0.4116	1.147	P > 0.05	ns
Sep vs Oct&Nov				
Seaweed species	Sep	Oct&Nov	Difference	95% CI of diff.
Fucus	9.937	10.08	0.1439	-1.029 to 1.317
Asco	7.144	7.839	0.6955	-0.5459 to 1.937
Seaweed species	Difference	t	P value	Summary
Fucus	0.1439	0.4009	P > 0.05	ns
Asco	0.6955	1.831	P > 0.05	ns

Annex 2. Two-way analysis of lipid content of *F. vesiculosus* and *A. nodosum* (Month*Seaweed)

Two-way ANOVA				
Source of Variation	% of total variation	P value		
Interaction	14.90	0.0129		
Seaweed	0.03	0.8510		
Month	17.03	0.0060		
Source of Variation	P value summary	Significant?		
Interaction	*	Yes		
Seaweed	ns	No		
Month	**	Yes		
Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	5	5.216	1.043	3.138
Seaweed	1	0.01182	0.01182	0.03555
Month	5	5.964	1.193	3.588
Residual	71	23.60	0.3324	
Number of missing values	25			
Bonferroni posttests				
Fucus vs Asco				
Month	Fucus	Asco	Difference	95% CI of diff.
May	1.485	2.019	0.5340	-0.3695 to 1.438
June	1.478	1.350	-0.1287	-0.8664 to 0.6090
July	1.340	1.805	0.4657	-0.4819 to 1.413
Aug	1.294	1.268	-0.02593	-0.9294 to 0.8776
Sep	1.343	1.283	-0.05966	-0.9632 to 0.8439
Oct&Nov	2.466	1.535	-0.9317	-1.669 to -0.1940
Month	Difference	t	P value	Summary
May	0.5340	1.604	P > 0.05	ns
June	-0.1287	0.4736	P > 0.05	ns
July	0.4657	1.334	P > 0.05	ns
Aug	-0.02593	0.07789	P > 0.05	ns
Sep	-0.05966	0.1792	P > 0.05	ns
Oct&Nov	-0.9317	3.428	P<0.01	**

Bonferroni posttests				
May vs June				
Seaweed species	May	June	Difference	95% CI of diff.
Fucus	1.485	1.478	-0.006721	-1.000 to 0.9866
Asco	2.019	1.350	-0.6695	-1.663 to 0.3239
Seaweed species	Difference	t	P value	Summary
Fucus	-0.006721	0.02212	P > 0.05	ns
Asco	-0.6695	2.203	P > 0.05	ns
May vs July				
Seaweed species	May	July	Difference	95% CI of diff.
Fucus	1.485	1.340	-0.1455	-1.234 to 0.9426
Asco	2.019	1.805	-0.2139	-1.355 to 0.9273
Seaweed species	Difference	t	P value	Summary
Fucus	-0.1455	0.4372	P > 0.05	ns
Asco	-0.2139	0.6126	P > 0.05	ns
May vs Aug				
Seaweed species	May	Aug	Difference	95% CI of diff.
Fucus	1.485	1.294	-0.1914	-1.280 to 0.8968
Asco	2.019	1.268	-0.7513	-1.839 to 0.3368
Seaweed species	Difference	t	P value	Summary
Fucus	-0.1914	0.5749	P > 0.05	ns
Asco	-0.7513	2.257	P > 0.05	ns
May vs Sep				
Seaweed species	May	Sep	Difference	95% CI of diff.
Fucus	1.485	1.343	-0.1425	-1.231 to 0.9456
Asco	2.019	1.283	-0.7362	-1.824 to 0.3520
Seaweed species	Difference	t	P value	Summary
Fucus	-0.1425	0.4281	P > 0.05	ns
Asco	-0.7362	2.212	P > 0.05	ns
May vs Oct&Nov				
Seaweed species	May	Oct&Nov	Difference	95% CI of diff.
Fucus	1.485	2.466	0.9811	-0.01219 to 1.974
Asco	2.019	1.535	-0.4845	-1.478 to 0.5088
Seaweed species	Difference	t	P value	Summary
Fucus	0.9811	3.229	P<0.01	**
Asco	-0.4845	1.595	P > 0.05	ns
June vs July				
Seaweed species	June	July	Difference	95% CI of diff.
Fucus	1.478	1.340	-0.1388	-1.132 to 0.8545
Asco	1.350	1.805	0.4556	-0.5957 to 1.507
Seaweed species	Difference	t	P value	Summary
Fucus	-0.1388	0.4568	P > 0.05	ns
Asco	0.4556	1.417	P > 0.05	ns
June vs Aug				
Seaweed species	June	Aug	Difference	95% CI of diff.
Fucus	1.478	1.294	-0.1846	-1.178 to 0.8087
Asco	1.350	1.268	-0.08185	-1.075 to 0.9115
Seaweed species	Difference	t	P value	Summary
Fucus	-0.1846	0.6076	P > 0.05	ns
Asco	-0.08185	0.2693	P > 0.05	ns
June vs Sep				
Seaweed species	June	Sep	Difference	95% CI of diff.
Fucus	1.478	1.343	-0.1358	-1.129 to 0.8575

Asco	1.350	1.283	-0.06671	-1.060 to 0.9266
Seaweed species	Difference	t	P value	Summary
Fucus	-0.1358	0.4468	P > 0.05	ns
Asco	-0.06671	0.2195	P > 0.05	ns
June vs Oct&Nov				
Seaweed species	June	Oct&Nov	Difference	95% CI of diff.
Fucus	1.478	2.466	0.9879	0.09939 to 1.876
Asco	1.350	1.535	0.1849	-0.7035 to 1.073
Seaweed species	Difference	t	P value	Summary
Fucus	0.9879	3.635	P<0.01	**
Asco	0.1849	0.6803	P > 0.05	ns
July vs Aug				
Seaweed species	July	Aug	Difference	95% CI of diff.
Fucus	1.340	1.294	-0.04583	-1.134 to 1.042
Asco	1.805	1.268	-0.5374	-1.679 to 0.6038
Seaweed species	Difference	t	P value	Summary
Fucus	-0.04583	0.1377	P > 0.05	ns
Asco	-0.5374	1.539	P > 0.05	ns
July vs Sep				
Seaweed species	July	Sep	Difference	95% CI of diff.
Fucus	1.340	1.343	0.003041	-1.085 to 1.091
Asco	1.805	1.283	-0.5223	-1.664 to 0.6190
Seaweed species	Difference	t	P value	Summary
Fucus	0.003041	0.009137	P > 0.05	ns
Asco	-0.5223	1.496	P > 0.05	ns
July vs Oct&Nov				
Seaweed species	July	Oct&Nov	Difference	95% CI of diff.
Fucus	1.340	2.466	1.127	0.1333 to 2.120
Asco	1.805	1.535	-0.2707	-1.322 to 0.7806
Seaweed species	Difference	t	P value	Summary
Fucus	1.127	3.708	P<0.001	***
Asco	-0.2707	0.8416	P > 0.05	ns
Aug vs Sep				
Seaweed species	Aug	Sep	Difference	95% CI of diff.
Fucus	1.294	1.343	0.04887	-1.039 to 1.137
Asco	1.268	1.283	0.01514	-1.073 to 1.103
Seaweed species	Difference	t	P value	Summary
Fucus	0.04887	0.1468	P > 0.05	ns
Asco	0.01514	0.04547	P > 0.05	ns
Aug vs Oct&Nov				
Seaweed species	Aug	Oct&Nov	Difference	95% CI of diff.
Fucus	1.294	2.466	1.172	0.1792 to 2.166
Asco	1.268	1.535	0.2668	-0.7266 to 1.260
Seaweed species	Difference	t	P value	Summary
Fucus	1.172	3.858	P<0.001	***
Asco	0.2668	0.8779	P > 0.05	ns
Sep vs Oct&Nov				
Seaweed species	Sep	Oct&Nov	Difference	95% CI of diff.
Fucus	1.343	2.466	1.124	0.1303 to 2.117
Asco	1.283	1.535	0.2516	-0.7417 to 1.245
Seaweed species	Difference	t	P value	Summary
Fucus	1.124	3.698	P<0.001	***
Asco	0.2516	0.8280	P > 0.05	ns

Annex 3. Two-way analysis of total polyphenol content of *F. vesiculosus* and *A. nodosum* (Month*Seaweed)

Two-way ANOVA				
Source of Variation	% of total variation	P value		
Interaction	9.25	0.0263		
Seaweed	2.27	0.0719		
Month	40.69	< 0.0001		
Source of Variation	P value summary	Significant?		
Interaction	*	Yes		
Seaweed	ns	No		
Month	***	Yes		
Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	5	13610	2722	2.718
Seaweed	1	3342	3342	3.337
Month	5	59894	11979	11.96
Residual	72	72110	1002	
Number of missing values	240			
Bonferroni posttests				
Fucus vs Asco				
Month	Fucus	Asco	Difference	95% CI of diff.
May	50.91	61.39	10.48	-55.55 to 76.51
June	44.44	36.14	-8.301	-62.21 to 45.61
July	33.83	86.73	52.89	-13.13 to 118.9
Aug	60.71	83.63	22.92	-43.11 to 88.95
Sep	100.7	124.8	24.12	-41.91 to 90.15
Oct&Nov	115.7	90.62	-25.03	-78.95 to 28.88
Month	Difference	t	P value	Summary
May	10.48	0.5735	P > 0.05	ns
June	-8.301	0.5564	P > 0.05	ns
July	52.89	2.895	P < 0.05	*
Aug	22.92	1.254	P > 0.05	ns
Sep	24.12	1.320	P > 0.05	ns
Oct&Nov	-25.03	1.678	P > 0.05	ns

Bonferroni posttests				
May vs Jun				
Seaweed	May	Jun	Difference	95% CI of diff.
Fucus	50.91	44.44	-6.464	-60.96 to 48.03
Asco	61.39	36.14	-25.24	-79.74 to 29.25
Seaweed	Difference	t	P value	Summary
Fucus	-6.464	0.3875	P > 0.05	ns
Asco	-25.24	1.514	P > 0.05	ns
May vs July				
Seaweed	May	July	Difference	95% CI of diff.
Fucus	50.91	33.83	-17.08	-76.77 to 42.62
Asco	61.39	86.73	25.34	-34.35 to 85.03
Seaweed	Difference	t	P value	Summary
Fucus	-17.08	0.9346	P > 0.05	ns
Asco	25.34	1.387	P > 0.05	ns
May vs Aug				
Seaweed	May	Aug	Difference	95% CI of diff.
Fucus	50.91	60.71	9.803	-49.89 to 69.50
Asco	61.39	83.63	22.24	-37.45 to 81.94
Seaweed	Difference	t	P value	Summary
Fucus	9.803	0.5365	P > 0.05	ns
Asco	22.24	1.217	P > 0.05	ns
May vs Sep				
Seaweed	May	Sep	Difference	95% CI of diff.
Fucus	50.91	100.7	49.81	-9.886 to 109.5
Asco	61.39	124.8	63.45	3.753 to 123.1
Seaweed	Difference	t	P value	Summary
Fucus	49.81	2.726	P < 0.05	*
Asco	63.45	3.472	P<0.01	**
May vs Oct&Nov				
Seaweed	May	Oct&Nov	Difference	95% CI of diff.
Fucus	50.91	115.7	64.75	10.25 to 119.2
Asco	61.39	90.62	29.23	-25.26 to 83.73
Seaweed	Difference	t	P value	Summary
Fucus	64.75	3.882	P<0.001	***
Asco	29.23	1.753	P > 0.05	ns
Jun vs July				
Seaweed	Jun	July	Difference	95% CI of diff.
Fucus	44.44	33.83	-10.61	-65.10 to 43.88
Asco	36.14	86.73	50.58	-3.909 to 105.1
Seaweed	Difference	t	P value	Summary
Fucus	-10.61	0.6363	P > 0.05	ns
Asco	50.58	3.033	P<0.01	**
Jun vs Aug				
Seaweed	Jun	Aug	Difference	95% CI of diff.
Fucus	44.44	60.71	16.27	-38.23 to 70.76
Asco	36.14	83.63	47.49	-7.005 to 102.0
Seaweed	Difference	t	P value	Summary
Fucus	16.27	0.9752	P > 0.05	ns
Asco	47.49	2.847	P < 0.05	*
Jun vs Sep				
Seaweed	Jun	Sep	Difference	95% CI of diff.
Fucus	44.44	100.7	56.27	1.779 to 110.8

Asco	36.14	124.8	88.69	34.20 to 143.2
Seaweed	Difference	t	P value	Summary
Fucus	56.27	3.374	P<0.01	**
Asco	88.69	5.317	P<0.001	***
Jun vs Oct&Nov				
Seaweed	Jun	Oct&Nov	Difference	95% CI of diff.
Fucus	44.44	115.7	71.21	22.47 to 119.9
Asco	36.14	90.62	54.48	5.740 to 103.2
Seaweed	Difference	t	P value	Summary
Fucus	71.21	4.773	P<0.001	***
Asco	54.48	3.652	P<0.001	***
July vs Aug				
Seaweed	July	Aug	Difference	95% CI of diff.
Fucus	33.83	60.71	26.88	-32.81 to 86.57
Asco	86.73	83.63	-3.096	-62.79 to 56.60
Seaweed	Difference	t	P value	Summary
Fucus	26.88	1.471	P > 0.05	ns
Asco	-3.096	0.1694	P > 0.05	ns
July vs Sep				
Seaweed	July	Sep	Difference	95% CI of diff.
Fucus	33.83	100.7	66.88	7.190 to 126.6
Asco	86.73	124.8	38.11	-21.59 to 97.80
Seaweed	Difference	t	P value	Summary
Fucus	66.88	3.661	P<0.001	***
Asco	38.11	2.086	P > 0.05	ns
July vs Oct&Nov				
Seaweed	July	Oct&Nov	Difference	95% CI of diff.
Fucus	33.83	115.7	81.82	27.33 to 136.3
Asco	86.73	90.62	3.896	-50.60 to 58.39
Seaweed	Difference	t	P value	Summary
Fucus	81.82	4.906	P<0.001	***
Asco	3.896	0.2336	P > 0.05	ns
Aug vs Sep				
Seaweed	Aug	Sep	Difference	95% CI of diff.
Fucus	60.71	100.7	40.00	-19.69 to 99.70
Asco	83.63	124.8	41.20	-18.49 to 100.9
Seaweed	Difference	t	P value	Summary
Fucus	40.00	2.189	P > 0.05	ns
Asco	41.20	2.255	P > 0.05	ns
Aug vs Oct&Nov				
Seaweed	Aug	Oct&Nov	Difference	95% CI of diff.
Fucus	60.71	115.7	54.94	0.4524 to 109.4
Asco	83.63	90.62	6.991	-47.50 to 61.48
Seaweed	Difference	t	P value	Summary
Fucus	54.94	3.294	P<0.01	**
Asco	6.991	0.4192	P > 0.05	ns
Sep vs Oct&Nov				
Seaweed	Sep	Oct&Nov	Difference	95% CI of diff.
Fucus	100.7	115.7	14.94	-39.55 to 69.43
Asco	124.8	90.62	-34.21	-88.70 to 20.28
Seaweed	Difference	t	P value	Summary
Fucus	14.94	0.8957	P > 0.05	ns
Asco	-34.21	2.051	P > 0.05	ns

Annex 4. Two-way analysis of yield of fucoidan from *F. vesiculosus* and *A. nodosum* (Month*Seaweed)

Two-way ANOVA				
Source of Variation	% of total variation	P value		
Interaction	19.28	< 0.0001		
Month	3.26	0.0265		
Seaweed	31.95	< 0.0001		
Source of Variation	P value summary	Significant?		
Interaction	***	Yes		
Month	*	Yes		
Seaweed	***	Yes		
Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	5	5.324	1.065	6.047
Month	1	0.9014	0.9014	5.119
Seaweed	5	8.822	1.764	10.02
Residual	76	13.38	0.1761	
Number of missing values	344			
Bonferroni posttests				
Fucus vs Asco				
Seaweed	Fucus	Asco	Difference	95% CI of diff.
May	3.320	3.851	0.5308	-0.2862 to 1.348
June	3.378	3.194	-0.1833	-0.8965 to 0.5298
July	2.880	4.117	1.237	0.3640 to 2.111
Aug	3.173	3.099	-0.07356	-0.9470 to 0.7998
Sep	2.825	2.626	-0.1984	-1.040 to 0.6432
Oct&Nov	2.854	2.779	-0.07463	-0.7697 to 0.6204
Seaweed	Difference	t	P value	Summary
May	0.5308	2.342	P > 0.05	ns
June	-0.1833	0.9268	P > 0.05	ns
July	1.237	5.108	P<0.001	***
Aug	-0.07356	0.3036	P > 0.05	ns
Sep	-0.1984	0.8499	P > 0.05	ns
Oct&Nov	-0.07463	0.3871	P > 0.05	ns

Bonferroni posttests				
May vs Jun				
Seaweed	May	Jun	Difference	95% CI of diff.
Fucus	3.320	3.378	0.05787	-0.6069 to 0.7227
Asco	3.851	3.194	-0.6563	-1.377 to 0.06479
Seaweed	Difference	t	P value	Summary
Fucus	0.05787	0.2838	P > 0.05	ns
Asco	-0.6563	2.967	P<0.01	**
May vs July				
Seaweed	May	July	Difference	95% CI of diff.
Fucus	3.320	2.880	-0.4403	-1.179 to 0.2986
Asco	3.851	4.117	0.2663	-0.5236 to 1.056
Seaweed	Difference	t	P value	Summary
Fucus	-0.4403	1.943	P > 0.05	ns
Asco	0.2663	1.099	P > 0.05	ns
May vs Aug				
Seaweed	May	Aug	Difference	95% CI of diff.
Fucus	3.320	3.173	-0.1468	-0.8857 to 0.5921
Asco	3.851	3.099	-0.7512	-1.541 to 0.03868
Seaweed	Difference	t	P value	Summary
Fucus	-0.1468	0.6479	P > 0.05	ns
Asco	-0.7512	3.101	P<0.01	**
May vs Sep				
Seaweed	May	Sep	Difference	95% CI of diff.
Fucus	3.320	2.825	-0.4951	-1.203 to 0.2130
Asco	3.851	2.626	-1.224	-2.014 to -0.4344
Seaweed	Difference	t	P value	Summary
Fucus	-0.4951	2.280	P > 0.05	ns
Asco	-1.224	5.053	P<0.001	***
May vs Oct&Nov				
Seaweed	May	Oct&Nov	Difference	95% CI of diff.
Fucus	3.320	2.854	-0.4662	-1.115 to 0.1828
Asco	3.851	2.779	-1.072	-1.793 to -0.3505
Seaweed	Difference	t	P value	Summary
Fucus	-0.4662	2.342	P < 0.05	*
Asco	-1.072	4.845	P<0.001	***
Jun vs July				
Seaweed	Jun	July	Difference	95% CI of diff.
Fucus	3.378	2.880	-0.4981	-1.219 to 0.2229
Asco	3.194	4.117	0.9226	0.2015 to 1.644
Seaweed	Difference	t	P value	Summary
Fucus	-0.4981	2.252	P > 0.05	ns
Asco	0.9226	4.172	P<0.001	***
Jun vs Aug				
Seaweed	Jun	Aug	Difference	95% CI of diff.
Fucus	3.378	3.173	-0.2047	-0.9258 to 0.5164
Asco	3.194	3.099	-0.09493	-0.8160 to 0.6262
Seaweed	Difference	t	P value	Summary
Fucus	-0.2047	0.9256	P > 0.05	ns
Asco	-0.09493	0.4292	P > 0.05	ns
Jun vs Sep				
Seaweed	Jun	Sep	Difference	95% CI of diff.
Fucus	3.378	2.825	-0.5529	-1.242 to 0.1365

Asco	3.194	2.626	-0.5680	-1.289 to 0.1530
Seaweed	Difference	t	P value	Summary
Fucus	-0.5529	2.615	P < 0.05	*
Asco	-0.5680	2.568	P < 0.05	*
Jun vs Oct&Nov				
Seaweed	Jun	Oct&Nov	Difference	95% CI of diff.
Fucus	3.378	2.854	-0.5240	-1.153 to 0.1046
Asco	3.194	2.779	-0.4153	-1.060 to 0.2296
Seaweed	Difference	t	P value	Summary
Fucus	-0.5240	2.718	P < 0.05	*
Asco	-0.4153	2.100	P > 0.05	ns
July vs Aug				
Seaweed	July	Aug	Difference	95% CI of diff.
Fucus	2.880	3.173	0.2934	-0.4965 to 1.083
Asco	4.117	3.099	-1.018	-1.807 to -0.2276
Seaweed	Difference	t	P value	Summary
Fucus	0.2934	1.211	P > 0.05	ns
Asco	-1.018	4.200	P<0.001	***
July vs Sep				
Seaweed	July	Sep	Difference	95% CI of diff.
Fucus	2.880	2.825	-0.05479	-0.8160 to 0.7064
Asco	4.117	2.626	-1.491	-2.281 to -0.7008
Seaweed	Difference	t	P value	Summary
Fucus	-0.05479	0.2347	P > 0.05	ns
Asco	-1.491	6.153	P<0.001	***
July vs Oct&Nov				
Seaweed	July	Oct&Nov	Difference	95% CI of diff.
Fucus	2.880	2.854	-0.02589	-0.7324 to 0.6806
Asco	4.117	2.779	-1.338	-2.059 to -0.6169
Seaweed	Difference	t	P value	Summary
Fucus	-0.02589	0.1195	P > 0.05	ns
Asco	-1.338	6.050	P<0.001	***
Aug vs Sep				
Seaweed	Aug	Sep	Difference	95% CI of diff.
Fucus	3.173	2.825	-0.3482	-1.109 to 0.4129
Asco	3.099	2.626	-0.4731	-1.263 to 0.3168
Seaweed	Difference	t	P value	Summary
Fucus	-0.3482	1.492	P > 0.05	ns
Asco	-0.4731	1.953	P > 0.05	ns
Aug vs Oct&Nov				
Seaweed	Aug	Oct&Nov	Difference	95% CI of diff.
Fucus	3.173	2.854	-0.3193	-1.026 to 0.3872
Asco	3.099	2.779	-0.3204	-1.041 to 0.4007
Seaweed	Difference	t	P value	Summary
Fucus	-0.3193	1.474	P > 0.05	ns
Asco	-0.3204	1.449	P > 0.05	ns
Sep vs Oct&Nov				
Seaweed	Sep	Oct&Nov	Difference	95% CI of diff.
Fucus	2.825	2.854	0.02890	-0.6453 to 0.7031
Asco	2.626	2.779	0.1527	-0.5684 to 0.8738
Seaweed	Difference	t	P value	Summary
Fucus	0.02890	0.1398	P > 0.05	ns
Asco	0.1527	0.6904	P > 0.05	ns

Annex 5. Two-way analysis of pure fucoidan content from *F. vesiculosus* and *A. nodosum* (Month*Seaweed)

Two-way ANOVA

Source of Variation	% of total variation	P value		
Interaction	14.68	0.0048		
Seaweed	6.67	0.0049		
Month	18.73	0.0008		

Source of Variation	P value summary	Significant?		
Interaction	**	Yes		
Seaweed	**	Yes		
Month	***	Yes		

Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	5	3.824	0.7647	3.692
Seaweed	1	1.738	1.738	8.391
Month	5	4.879	0.9758	4.711
Residual	77	15.95	0.2071	

Number of missing values	55

Bonferroni posttests

Fucus vs Asco

Month	Fucus	Asco	Difference	95% CI of diff.
May	2.885	3.022	0.1367	-0.5289 to 0.8023
June	2.750	2.984	0.2344	-0.3466 to 0.8154
July	2.557	3.788	1.232	0.5201 to 1.943
Aug	2.578	2.833	0.2550	-0.4566 to 0.9666
Sep	2.560	2.322	-0.2383	-0.9240 to 0.4474
Oct&Nov	2.515	2.610	0.09545	-0.4585 to 0.6494

Month	Difference	t	P value	Summary
May	0.1367	0.5560	P > 0.05	ns
June	0.2344	1.093	P > 0.05	ns
July	1.232	4.687	P<0.001	***
Aug	0.2550	0.9705	P > 0.05	ns
Sep	-0.2383	0.9413	P > 0.05	ns
Oct&Nov	0.09545	0.4666	P > 0.05	ns

Bonferroni posttests				
May vs June				
Seaweed	May	June	Difference	95% CI of diff.
Fucus	2.885	2.750	-0.1350	-0.8557 to 0.5857
Asco	3.022	2.984	-0.03722	-0.8189 to 0.7444
Seaweed	Difference	t	P value	Summary
Fucus	-0.1350	0.6105	P > 0.05	ns
Asco	-0.03722	0.1552	P > 0.05	ns
May vs July				
Seaweed	May	July	Difference	95% CI of diff.
Fucus	2.885	2.557	-0.3283	-1.129 to 0.4726
Asco	3.022	3.788	0.7667	-0.08959 to 1.623
Seaweed	Difference	t	P value	Summary
Fucus	-0.3283	1.336	P > 0.05	ns
Asco	0.7667	2.918	P<0.01	**
May vs Aug				
Seaweed	May	Aug	Difference	95% CI of diff.
Fucus	2.885	2.578	-0.3067	-1.108 to 0.4943
Asco	3.022	2.833	-0.1883	-1.045 to 0.6679
Seaweed	Difference	t	P value	Summary
Fucus	-0.3067	1.248	P > 0.05	ns
Asco	-0.1883	0.7168	P > 0.05	ns
May vs Sep				
Seaweed	May	Sep	Difference	95% CI of diff.
Fucus	2.885	2.560	-0.3250	-1.093 to 0.4426
Asco	3.022	2.322	-0.7000	-1.556 to 0.1563
Seaweed	Difference	t	P value	Summary
Fucus	-0.3250	1.380	P > 0.05	ns
Asco	-0.7000	2.664	P < 0.05	*
May vs Oct				
Seaweed	May	Oct	Difference	95% CI of diff.
Fucus	2.885	2.515	-0.3705	-1.060 to 0.3187
Asco	3.022	2.610	-0.4117	-1.193 to 0.3700
Seaweed	Difference	t	P value	Summary
Fucus	-0.3705	1.752	P > 0.05	ns
Asco	-0.4117	1.716	P > 0.05	ns
June vs July				
Seaweed	June	July	Difference	95% CI of diff.
Fucus	2.750	2.557	-0.1933	-0.9750 to 0.5883
Asco	2.984	3.788	0.8039	0.02223 to 1.586
Seaweed	Difference	t	P value	Summary
Fucus	-0.1933	0.8060	P > 0.05	ns
Asco	0.8039	3.351	P<0.01	**
June vs Aug				
Seaweed	June	Aug	Difference	95% CI of diff.
Fucus	2.750	2.578	-0.1717	-0.9533 to 0.6100
Asco	2.984	2.833	-0.1511	-0.9328 to 0.6305
Seaweed	Difference	t	P value	Summary
Fucus	-0.1717	0.7157	P > 0.05	ns
Asco	-0.1511	0.6300	P > 0.05	ns
June vs Sep				
Seaweed	June	Sep	Difference	95% CI of diff.
Fucus	2.750	2.560	-0.1900	-0.9374 to 0.5574
Asco	2.984	2.322	-0.6628	-1.444 to 0.1189
Seaweed	Difference	t	P value	Summary
Fucus	-0.1900	0.8284	P > 0.05	ns
Asco	-0.6628	2.763	P < 0.05	*
June vs Oct				
Seaweed	June	Oct	Difference	95% CI of diff.
Fucus	2.750	2.515	-0.2355	-0.9021 to 0.4311
Asco	2.984	2.610	-0.3744	-1.074 to 0.3247

Seaweed	Difference	t	P value	Summary
Fucus	-0.2355	1.151	P > 0.05	ns
Asco	-0.3744	1.745	P > 0.05	ns

July vs Aug				
Seaweed	July	Aug	Difference	95% CI of diff.
Fucus	2.557	2.578	0.02167	-0.8346 to 0.8779
Asco	3.788	2.833	-0.9550	-1.811 to -0.09874

Seaweed	Difference	t	P value	Summary
Fucus	0.02167	0.08246	P > 0.05	ns
Asco	-0.9550	3.635	P<0.01	**

July vs Sep				
Seaweed	July	Sep	Difference	95% CI of diff.
Fucus	2.557	2.560	0.003333	-0.8218 to 0.8284
Asco	3.788	2.322	-1.467	-2.323 to -0.6104

Seaweed	Difference	t	P value	Summary
Fucus	0.003333	0.01316	P > 0.05	ns
Asco	-1.467	5.582	P<0.001	***

July vs Oct				
Seaweed	July	Oct	Difference	95% CI of diff.
Fucus	2.557	2.515	-0.04212	-0.7948 to 0.7106
Asco	3.788	2.610	-1.178	-1.960 to -0.3967

Seaweed	Difference	t	P value	Summary
Fucus	-0.04212	0.1824	P > 0.05	ns
Asco	-1.178	4.913	P<0.001	***

Aug vs Sep				
Seaweed	Aug	Sep	Difference	95% CI of diff.
Fucus	2.578	2.560	-0.01833	-0.8434 to 0.8068
Asco	2.833	2.322	-0.5117	-1.368 to 0.3446

Seaweed	Difference	t	P value	Summary
Fucus	-0.01833	0.07241	P > 0.05	ns
Asco	-0.5117	1.947	P > 0.05	ns

Aug vs Oct				
Seaweed	Aug	Oct	Difference	95% CI of diff.
Fucus	2.578	2.515	-0.06379	-0.8165 to 0.6889
Asco	2.833	2.610	-0.2233	-1.005 to 0.5583

Seaweed	Difference	t	P value	Summary
Fucus	-0.06379	0.2762	P > 0.05	ns
Asco	-0.2233	0.9311	P > 0.05	ns

Sep vs Oct				
Seaweed	Sep	Oct	Difference	95% CI of diff.
Fucus	2.560	2.515	-0.04545	-0.7625 to 0.6716
Asco	2.322	2.610	0.2883	-0.4933 to 1.070

Seaweed	Difference	t	P value	Summary
Fucus	-0.04545	0.2066	P > 0.05	ns
Asco	0.2883	1.202	P > 0.05	ns