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Antidiabetic properties of polysaccharide- and polyphenolic-enriched fractions from the brown seaweed *Ascophyllum nodosum*¹

Junzeng Zhang, Christa Tiller, Jingkai Shen, Can Wang, Gabrielle S. Girouard, Dorothy Dennis, Colin J. Barrow, Mingsan Miao, and H. Stephen Ewart

Abstract: We screened seaweed species from Atlantic Canada for antidiabetic activity by testing extracts for α -glucosidase inhibitory effect and glucose uptake stimulatory activity. An aqueous ethanolic extract of *Ascophyllum nodosum* was found to be active in both assays, inhibiting rat intestinal α -glucosidase ($IC_{50} = 77 \mu\text{g/mL}$) and stimulating basal glucose uptake into 3T3-L1 adipocytes during a 20-minute incubation by about 3-fold (at 400 $\mu\text{g/mL}$ extract). Bioassay-guided fractionation of the *A. nodosum* extract showed that α -glucosidase inhibition was associated with polyphenolic components in the extract. These polyphenolics, along with other constituents appeared to be responsible for the stimulatory activity on glucose uptake. However, attempts to further concentrate this activity through fractionation techniques were unsuccessful. A crude polyphenol extract (PPE), an enriched polyphenolic fraction (PPE-F1) and a polysaccharide extract (PSE) were prepared from commercial *A. nodosum* powder and administered to streptozotocin-diabetic mice for up to 4-weeks by daily gavage at 200 mg/kg body mass. PPE and PPE-F1 improved fasting serum glucose level in diabetic mice; however, the effect was only statistically significant at day 14. In addition, PPE-F1 was shown to blunt the rise in blood glucose after an oral sucrose tolerance test in diabetic mice. Mice treated with PPE and PPE-F1 had decreased blood total cholesterol and glycated serum protein levels compared with untreated diabetic mice, whereas PPE also normalized the reduction in liver glycogen level that occurred in diabetic animals. All 3 *A. nodosum* preparations improved blood antioxidant capacity.

Key words: seaweed, *Ascophyllum nodosum*, α -glucosidase, diabetes.

Résumé : On a établi une recherche d'un produit anti-diabétique, parmi les algues locales de la région Atlantique du Canada, en examinant la capacité d'un effet inhibiteur de l'enzyme α -glucosidase et une stimulation de l'incorporation cellulaire du glucose. Un extrait éthanol-aqueux de *Ascophyllum nodosum* nous a donné une activité positive chez les deux essais, une inhibition de l' α -glucosidase provenant de l'intestin du rat ($IC_{50} = 77 \mu\text{g/mL}$) et puis une stimulation triple, à une concentration de 400 $\mu\text{g/mL}$, de l'incorporation du glucose dans les adipocytes 3T3-L1 durant une période de 20 minutes. L'extrait de *A. nodosum* a été divisé, guidé par les résultats biologiques, et a ainsi démontré la présence d'éléments polyphénoliques associé à l'inhibition de l' α -glucosidase. Ces éléments polyphénoliques ainsi que d'autres semblent être responsables de l'incorporation stimulée du glucose. Il a été impossible de raffiner cette activité lors d'une division des composants. Un extrait brut polyphénolique (PPE), un extrait enrichi polyphénolique (PPE-F1) et puis un extrait polysaccharide (PSE) furent préparés d'une poudre commerciale de *A. nodosum* et utilisés dans une étude utilisant des souris, rendues diabétiques par injections de streptozotocin, traitées par un gavage journalier de l'extrait 200 mg/kg du poids corporel durant une période de 4 semaines. Le taux du glucose sanguin à jeun des souris fut moins élevé en présence des extraits qu'en leurs absence. Cependant, l'effet était seulement significatif au jour 14. Les résultats étant toutefois variables. En plus, lors d'un essai oral de la tolérance au sucrose chez la souris diabétique, l'extrait PPE-F1 a empêché l'augmentation du taux du glucose sanguin. Les extraits PPE et PPE-F1 ont réduit le taux de cholestérol sanguin et les niveaux de glycation des protéines en comparaison de ces niveaux en absence des extraits tandis que l'extrait PPE a présenté une réduction du niveau de glycogène du foie chez les souris diabétiques. Les trois extraits de *A. nodosum* ont tous amélioré la capacité antioxydante du sang.

Mots-clés : algues, *Ascophyllum nodosum*, α -glucosidase, diabète.

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Introduction

Diabetes mellitus is a metabolic disorder characterized by hyperglycemia that results from absolute or relative deficiency of insulin secretion, impaired insulin action, or both (ADA Position Statement 2003). Type II diabetes, the most common form of the disease, is typically preceded by a period of “pre-diabetes”, in which fasting glucose levels are slightly above normal and (or) postprandial glucose clearance from the blood is impaired (Baron 1998; Schwartz 2006). Excessive postprandial hyperglycemia is associated with oxidative stress and may accelerate the onset of diabetes and its vascular complications (Baron 1998; Ceriello et al. 2006). Thus certain antidiabetic therapies that limit blood glucose excursions after a meal, such as the use of α -glucosidase inhibitors, have particular relevance in the prevention and control of diabetes.

Intestinal α -glucosidase (EC3.2.1.20) cleaves glucose and other monosaccharides from starch and sucrose, the major carbohydrate constituents in food, for absorption into the bloodstream. Inhibition of α -glucosidase by acarbose (Precose) or miglitol (Glycet) delays the absorption of glucose from the small intestine (Bischoff 1994; Lebovitz 1997) and, as demonstrated in the STOP-NIDDM trial (Chiasson et al. 2002), is an effective antidiabetic therapeutic approach. Research has also revealed that a variety of food materials contain compounds that inhibit α -glucosidase including fermented soybean (Fujita et al. 2001), purple sweet potato (Matsui et al. 2001), propolis (Matsui et al. 2004), leaf tea (Youn et al. 2004), fish protein hydrolysates (Matsui et al. 1999) and seaweed (Kurihara et al. 1999). Extracts prepared from these sources effectively blunt the appearance of glucose in blood after carbohydrate tolerance testing in animals and, in the case of fermented soybean extract, in a humans (Fujita et al. 2001). Thus, α -glucosidase inhibitory constituents from foods show promise, potentially as dietary supplements or functional food ingredients, that could help maintain healthy blood glucose levels.

We screened seaweed species of Atlantic Canada for anti-diabetic activity by testing extracts in 2 primary bioassay screens: α -glucosidase inhibitory activity and stimulation of glucose uptake into 3T3-L1 fat cells. An extract from *Ascophyllum nodosum* (knotted wrack; Norwegian kelp), was particularly intriguing since it was active in both assay screens. In the present study, we report on these activities and subsequent testing of *A. nodosum* preparations in diabetic mice.

Materials and methods

Materials

Fresh *A. nodosum* (L.) Le Jolis was collected along the southern shore of Nova Scotia, Canada. Commercial *A. nodosum* powder (40 Mesh, denoted ASCO-40) was a gift from Acadian Seaplants Ltd. (Dartmouth, N.S.). The 3T3-L1 preadipocyte cell line was acquired from ATCC (Bethesda, Md.). Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, fetal bovine serum, and calf serum were purchased from Invitrogen Corp. (Burlington, Ont.). Rat intestinal powder, 2-deoxyglucose, cytochalasin B, *p*-nitrophenyl glucoside, castanospermine, streptozotocin, 1-methyl-3-isobutylxanthine (IBMX), dexa-

methasone, and insulin were from Sigma-Aldrich Canada Ltd. (Oakville, Ont.). [3 H]deoxyglucose was from MP Bio-medicals (Irvine, Calif.). Acarbose was from Bayer Pharmaceutical and Healthcare Ltd. (Beijing, China); carboxymethylcellulose was from by Hua Long Reagent Co. (Tianjin, China); metformin hydrochloride tablets were manufactured by Xin Yi Pharmaceutical Co., Shanghai Pharm Group Ltd. (Shanghai, China); glucose and cholesterol assay kits were supplied by Zhejiang Dong Ou Biological Engineering Ltd. (Wenzhou, China); alkaline phosphatase, glycosylated serum protein, and blood total antioxidant indices were measured by using assay kits from Nanjing Jian Cheng Biological Engineering Ltd. (Nanjing, China). All other chemicals were reagent grade.

Experimental animals

Male mice weighing 18–20 g were used in the studies. For the initial animal testing, mice of the C57BL/6J strain were obtained from Charles River Canada (St. Constant, Que.). For subsequent work, mice of the Kunming strain were obtained from Hebei Experimental Animal Center (Shijiazhuang, China). These studies were carried out in full compliance with regulations of the Canadian Council on Animal Care and the Guiding Principles of China Council on Animal Care.

Preparation of crude extract and fractions from *A. nodosum*

Fresh *A. nodosum* was washed with distilled water, freeze-dried, and milled by using a kitchen grinder. This material (160 g) was extracted with 2 L of 50% ethanol twice under refluxing (80 °C) for 90 min. The aqueous ethanol extracts were pooled, concentrated on a rotatory evaporator at 40 °C, and freeze-dried to yield 39.8 g of 50% aqueous ethanol extract (ON-E1). ON-E1 was suspended in 200 mL water, partitioned between ethyl acetate (3 × 300 mL), and then *n*-butanol (2 × 300 mL). The 2 organic extracts were combined and dried on a rotatory evaporator to yield ON-F1 (13.3 g). ON-F1 was then loaded on a C18 column and eluted sequentially with water (600 mL) and water-methanol (1:1, 800 mL). The water-methanol elution was dried to give fraction ON-F2 (5.9 g). ON-E1 and its fractions were used for initial screening for α -glucosidase inhibitory activity. For subsequent work we used dried, milled *A. nodosum*. (ASCO-40).

Two hundred grams of ASCO-40 powder was extracted with 1.2 L 50% aqueous ethanol at 50 °C for 2 h. After evaporating to dryness, 32.9 g of crude aqueous ethanol extract (AS-E1) was obtained. For animal studies, polyphenolic and polysaccharide-enriched fractions were prepared. The crude polyphenolic extract and an enriched fraction were obtained from 2 kg of ASCO-40 powder extracted with 12 L 50% ethanol at 50 °C under stirring for 2 h, and the liquid extract was separated by centrifugation and concentrated to 3.1 L on a rotatory evaporator at 40 °C. This liquid extract was spray-dried to yield a crude polyphenol extract (PPE, 203 g). PPE was then suspended in water and loaded on an HP-20 column (batch purification, 1:15, *m/m*). Water, 50% ethanol, and ethanol were used to elute the column sequentially, and the 50% ethanol fraction was on a rotatory evaporator at 40 °C and freeze-dried to obtain a

Table 1. α -Glucosidase inhibition and total polyphenol content of an aqueous ethanol extract and fractions from *Ascophyllum nodosum*.

	α -Glucosidase assay IC ₅₀ , μ g/mL	Total phenol content, PGE%
ON-E1	77.0	22.5
ON-F1	38.0	39.8
ON-F2	24.0	70.2

Note: PGE%, phloroglucinol equivalent. ON-E1 is an aqueous ethanol extract. It was extracted with ethyl acetate and then *n*-butanol and these 2 organic extractions combined to yield ON-F1. ON-F1 was then loaded on a C18 column and eluted sequentially with water and water-methanol. The water-methanol elution was dried to give ON-F2. IC₅₀ values were determined by measuring inhibitory activity at 5 or 6 concentrations each performed in duplicate. Total polyphenolic content of each sample was performed in duplicate.

purified polyphenolic fraction (PPE-F1, 28 g by pooling all batches together). The polyphenolic content of PPE and PPE-F1 in percentage phloroglucinol equivalents was 29.5% and 65.7%, respectively. An enriched polysaccharide fraction was prepared from 300 g ASCO-40 powder extracted with 2.4 L water at 80 °C for 2 h under stirring. The filtrated liquid water extract was then mixed with 4 volumes of 95% ethanol, stirred, and the precipitate obtained by centrifugation. This precipitate was freeze-dried to give a crude polysaccharide fraction (PSE, 18.3 g).

Polyphenol content

Total polyphenol content of samples was measured by the Folin-Ciocalteu method (Singleton et al. 1999) modified for use with a 96-well microplate (Zhang et al. 2006). Samples were run in duplicate.

Assay of α -glucosidase

The activity of α -glucosidase was measured according to Sawada et al. (1993) by using mammalian α -glucosidase (Type II), extracted from rat intestinal powder. The assay is based on the hydrolysis of *p*-nitrophenyl glucoside, an artificial substrate for α -glucosidase, which results in an increase in absorbance at 400 nm. Extracts were screened for their ability to impair this activity. Castanospermine was used as a positive control. The half maximal inhibitory concentration, IC₅₀, of lead extract/fractions was determined by measuring inhibitory activity at 5 or 6 concentrations, each performed in duplicate. Curve-fitting was performed with SoftMax Pro software (Molecular Devices Corp., Sunnyvale, Calif.).

Cell culture

3T3-L1 preadipocytes were maintained in Dulbecco's modified Eagle's medium containing 25 mmol/L glucose (DMEM) plus 10% calf serum, 1% antibiotic (10 000 U penicillin, 10 mg streptomycin), and 2 mmol/L glutamine. For experiments, cells were grown to confluency in 12-well plates at which time they were treated with differentiation medium (DMEM plus 10% fetal bovine serum (FBS), antibiotic, glutamine, 10 mg/mL insulin, 0.5 mmol/L IBMX, and 0.3 μ g/mL dexamethasone) for 3 days. Thereafter, cells were treated with differentiation medium without the hor-

mon additives and were used 10–12 days from the start of the differentiation protocol.

Assay of glucose transport

3T3-L1 cells were incubated overnight in DMEM containing 0.2% FBS. Cells were washed once in PBS then incubated for 20 min in fresh medium without or with insulin (5 nmol/L) and containing, or not, various concentrations of *A. nodosum* extract (ON-E1). When added, ON-E1 was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 100 mg/mL so that at the highest concentration of ON-E1 tested (400 μ g/mL) the amount of DMSO in the incubation was 4 μ L/mL of DMEM. This amount of DMSO was present in each of the incubations and added as vehicle to control incubations. The treatment was stopped by removal of medium followed by a wash step with 1 mL of uptake buffer (phosphate buffered saline (PBS), pH 7.4, 0.5 mmol/L MgCl₂, 1 mmol/L CaCl₂, and 2.5 mmol/L KCl). Cells were then incubated in uptake buffer containing 50 μ mol/L deoxyglucose (50 μ mol/L) and [³H]deoxyglucose (1 μ Ci/mL). Nontransporter-mediated 2-deoxyglucose uptake was determined in parallel in the presence of cytochalasin B (75 μ mol/L) and subtracted from both basal and stimulated uptake measurements. The uptake was stopped after 10 min by aspiration of the uptake solution and rapidly washing the wells twice with 1 mL ice-cold 0.9% NaCl. Cells were lysed in 0.05M NaOH (1 mL/well) and 800 μ L of the lysate was mixed with 3 mL of scintillation fluid and radioactivity determined. Cell protein content was determined by the Coomassie protein assay with bovine serum albumin as standard.

Streptozotocin-diabetic mouse studies

Two diabetic mouse models were used. For the initial animal testing, mice (C57BL/6J strain) were made type II diabetic by using a procedure modified from Manchem et al. (2001). Mice were fed for 2–3 weeks on a high fat/high sucrose diet (No. D12451, Research Diets Inc., New Brunswick, N.J.). They were then injected i.p. with streptozotocin (35 mg/kg body mass dissolved in PBS) daily for 3 consecutive days. Before each injection, mice were deprived of food for 2–3 h. Blood glucose was measured with a glucometer (LifeScan Inc., Milpitas, Calif.) by using a drop of blood from the saphenous vein. Mice with fasting blood glucose levels between 12 and 20 mmol/L were used in the study.

Before tolerance testing mice were deprived of food for 2 h and then gavaged with ON-F2 (300 mg/kg body mass suspended in 0.25 mL PBS) or 0.25 mL PBS (controls). Five to 10 min later they were challenged with 2 g of maltose/kg body mass delivered via gavage (0.25 mL of a 20% solution/25 g mouse). A drop of blood was taken from the saphenous vein just before (0 time) and at 10, 30, 60, and 120 min after administration of the challenge.

For subsequent studies, male mice of the Kunming strain were deprived of food for 12 h and then given STZ (110 mg/kg body mass) dissolved in sodium citrate buffer via tail vein. After 8 days, mice with blood glucose levels between 11 to 20 mmol/L were entered in the study. Diabetic animals were divided into 5 groups (10 mice per group). Of these, 3 groups were treated with *A. nodosum*

Table 2. The effect of *Ascophyllum nodosum* extracts and fractions on body mass in STZ-diabetic mice.

Group	n	Dose, mg/kg	Day				
			0	7	14	21	28
Nondiabetic	10	—	19.9±0.3	28.1±0.4*	35.5±0.5*	38.0±0.9*	38.4±1.0*
STZ-diabetic	10	—	19.6±0.5	21.8±1.0	24.1±1.1	23.3±1.1	22.2±1.1
Metformin	10	250	18.8±0.4	20.0±1.3	23.1±1.6	22.9±1.5	20.7±1.4
PPE	10	200	19.5±0.4	21.0±0.8	22.8±1.0	21.8±1.1	20.9±0.9
PSE	10	200	18.8±0.7	22.3±1.2	26.0±1.3	25.1±1.3	23.8±1.3
PPE-F1	10	200	19.2±0.4	20.1±0.9	21.7±1.2	21.1±1.0	20.1±1.1

Note: Mice (Kunming strain) were made diabetic with a single injection of STZ (110 mg/kg body mass) and gavaged daily for 4 weeks with vehicle (STZ-diabetic group), metformin (250 mg/kg body mass), or 200 mg/kg body mass of *A. nodosum* constituents (a crude polyphenol extract, PPE group; a crude polysaccharide extract, PSE group; or a polyphenol-enriched fraction, PPE-F1 group). Mice not injected with STZ (nondiabetic group) were used for comparison. Values are means ± SE of body mass in grams. *, significantly different by Bonferroni multiple comparison test at $p < 0.05$ vs. corresponding value of the STZ-diabetic group.

samples (PPE, PPE-F1, and PSE), one group was a control group, receiving vehicle alone, and one group received metformin, an oral antidiabetic agent. A 6th group, consisting of nondiabetic mice, was also included for comparison. They were treated with vehicle alone. Mice were housed 5 to a cage with free access to food and water.

Animals were treated daily by gavage for 4 weeks. Samples were dissolved in carboxymethylcellulose (CMC; 0.5%; distilled water) at a concentration of 20 mg/mL and delivered at a dose of 200 mg/kg body mass. The control groups were gavaged with distilled water (0.2 mL/20 g mouse). Metformin was gavaged at a dose of 250 mg/kg in 0.2 mL distilled water.

Animals were monitored for general activity on a daily basis. Body mass, food and water consumption were measured weekly. Fasting blood glucose level was measured on days 0, 7, 14, and 28 (from tail vein blood) between 09:00 and 10:00. On day 28, blood was collected from the orbital vein for blood antioxidant activity, serum total cholesterol, and glycated serum protein determinations. Animals were killed and tissues removed. Liver was removed for glycogen determination. Liver, kidney, and pancreas tissue slices were examined by optical microscopy for signs of cell damage.

Sucrose challenge

Diabetic mice treated with a single dose of PPE-F1 (50, 100, or 200 mg/kg body mass), acarbose (25 mg/kg body mass) or saline (0.2 mL/20 g mouse; controls) by gavage. For comparison, a group of nondiabetic animals were treated saline (0.2 mL/20 g mouse). Ten minutes after gavaging of samples, blood was sampled from the tail vein for glucose measurement (time 0). The animals were then gavaged with sucrose (20% in water at a dose of 2 g/kg body mass). Blood was sampled from the tail vein at 10, 30, 60, and 120 min after the sucrose challenge for glucose measurement.

Biochemical analyses

Total cholesterol

Serum (100 µL) was treated with cholesterol esterase to hydrolyze the cholesterol esters to cholesterol. Total cholesterol was determined by using a colorimetric method that involved the conversion of cholesterol to 4-cholesten-3-one

and H₂O₂ using cholesterol oxidase followed by reaction of the H₂O₂ with 4-aminoantipyrine in the presence of phenol and peroxidase to give red quinone that was monitored at 505 nm.

Glycated protein

Glycated protein in serum (100 µL) was determined by using a commercial kit based on the ability of ketoamines to reduce nitroblue tetrazolium (NBT) to diformazan. Formazan blue formation was monitored at 530 nm.

Antioxidant activity

The antioxidant capacity of serum (100 µL) was determined by using a commercial kit based on the NBT method (Sun et al. 1988). In the assay, superoxide radicals generated by the xanthine oxidase system reduce NBT to diformazan. In the presence of antioxidants less formazan blue forms resulting in reduction in absorbance.

Liver glycogen

Liver (200 mg) was homogenized in 5% trichloroacetic acid (4 mL) and left at 4 °C overnight then centrifuged at 3000 rpm at 4 °C for 10 min. The supernatant was assayed for glycogen with anthrone reagent (Carroll et al. 1955).

Liver alkaline phosphatase

Liver tissue (200 mg) was homogenized in saline to produce a 1% solution (mg/mL) and then centrifuged at 3000 r/min (1100g) for 10 min at low temperature. The supernatant was assayed for alkaline phosphatase (ALP) by using a commercial kit based on the 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium (NBT) substrate system. Formazan level was monitored at 520 nm. Liver supernatant protein content was determined by using the coomassie blue reagent with serum albumin as standard.

Statistics

Results are expressed as means ± SE. Data were analysed by using two-way (repeated measures) ANOVA or one-way ANOVA (data in Table 4) with GraphPad Prism software (San Diego, Calif.). Multiple comparison testing was performed by using the Bonferroni test or Dunnett's test, with statistical significance corresponding to $p < 0.05$.

Table 3. The effect of *Ascophyllum nodosum* extracts and fractions on blood glucose of the STZ-diabetic mice.

Group	n	Dose, mg/kg	Day				
			0	7	14	21	28
Nondiabetic	10	—	5.71±0.28*	5.91±0.18*	4.92±0.23*	5.59±0.28*	6.13±0.33*
STZ-diabetic	10	—	15.02±1.00	16.61±1.14	17.03±0.91	16.24±0.84	22.05±0.93
Metformin	10	250	13.50±0.84	14.51±1.53	13.07±0.63*	13.29±0.55	16.31±1.70*
PPE	10	200	15.09±0.93	12.78±1.25	12.43±0.86*	13.41±1.06	21.13±1.54
PSE	10	200	13.04±0.86	13.47±0.81	14.46±0.52	16.82±1.44	20.50±1.79
PPE-F1	10	200	14.42±0.99	14.64±1.24	12.82±0.58*	14.83±1.75	18.61±1.79

Note: Mice (Kunming strain) were made diabetic with a single inject of STZ (110 mg/kg body mass) and gavaged daily for 4 weeks with vehicle (STZ-diabetic group), metformin, a crude polyphenol extract (PPE group), a crude polysaccharide extract (PSE group), or a polyphenol-enriched fraction (PPE-F1 group). Mice not injected with STZ (nondiabetic group) were used for comparison. Animals were fasted overnight and blood for glucose measurement was obtained from a tail vein. Results are means ± SE of blood glucose (mmol/L). *, significantly different by Bonferroni multiple-comparison test at $p < 0.05$ vs. the STZ-diabetic group.

Table 4. The effect of *Ascophyllum nodosum* extracts and fractions on the biochemical parameters in STZ-diabetic mice.

Group	n	Dose, mg/kg	Total cholesterol, mmol/L	Glycated serum protein, mmol/L	Liver glycogen, mg/g wet tissue	Antioxidant activity, U/mL serum	Liver ALP, U/g protein
Nondiabetic	10	—	2.16±0.15	4.94±0.14*	6.70±0.34*	6.92±0.38	66.00±0.65
STZ-diabetic	10	—	2.67±0.12	6.20±0.18	4.41±0.29	5.50±0.38	68.68±0.95
Metformin	10	250	1.93±0.22*	5.22±0.17*	5.51±0.26	8.15±0.57*	68.93±1.93
PPE	10	200	1.99±0.24*	5.15±0.14*	6.49±0.51*	8.54±0.41*	74.73±1.63*
PSE	10	200	2.64±0.16	6.23±0.21	5.57±0.50	7.55±0.51*	78.85±1.57*
PPE-F1	10	200	2.00±0.14*	5.47±0.19*	4.60±0.39	7.56±0.13*	69.07±1.66

Note: ALP, alkaline phosphatase. Mice (Kunming strain) were made diabetic with a single injection of STZ (110 mg/kg body mass) and gavaged daily for 4-week with vehicle (STZ-diabetic group), metformin, a crude polyphenol extract (PPE group), a crude polysaccharide extract (PSE group) or a polyphenol-enriched fraction (PPE-F1 group). Mice not injected with STZ (Nondiabetic group) were used for comparison. At the end of the treatment period, the animals were fasted overnight and blood was collected from the orbital vein. Results are means ± SE. *, significant difference by Dunnett's multiple comparison test at $p < 0.05$ vs. the STZ-diabetic group.

Results

An aqueous alcohol extract of *A. nodosum* (ON-E1) inhibited α -glucosidase in vitro with an IC_{50} of about 77 μ g/mL (Table 1). The potency of the α -glucosidase inhibition was increased with subsequent fractionation, which was positively correlated with the polyphenolic content of the fractions (Table 1).

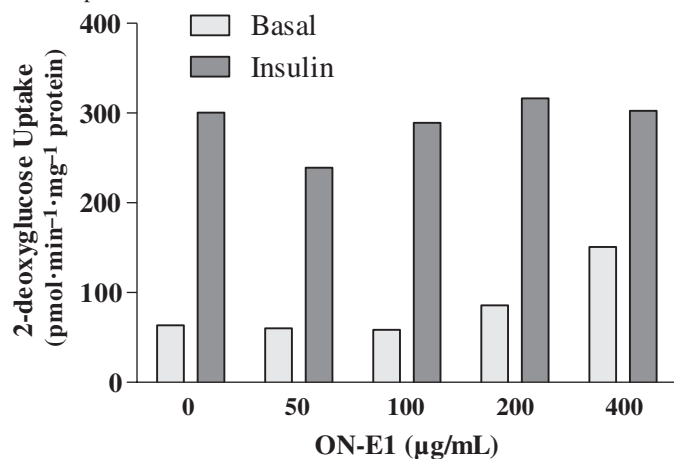
ON-E1 stimulated basal glucose uptake into 3T3-L1 cells (Fig. 1). The basal glucose uptake rate was increased from 63.4 $\text{pmol}\cdot\text{min}^{-1}\cdot\text{mg cell protein}^{-1}$ to 85.8 and 150.9 $\text{pmol}\cdot\text{min}^{-1}\cdot\text{mg cell protein}^{-1}$ at 200 and 400 μ g/mL ON-E1, respectively. ON-E1 (400 μ g/mL) was nontoxic to cells, as determined by the neutral red assay (results not shown). When cells were co-treated with insulin (5 nmol/L) ON-E1 did not increase glucose uptake above the insulin-stimulated rate. Various attempts to further purify this activity using bioassay-guided fraction procedures were unsuccessful.

To determine whether the α -glucosidase inhibitory activity might be relevant in vivo, a maltose challenge in type II diabetic mice (C57BL/6J strain) testing ON-F2 was carried out (Fig. 2). While the differences between ON-F2-treated and control were not statistically significant at any of the time points (two-way ANOVA) there was a trend of reduced glycemia in the treatment group. This led us to do a further, more comprehensive animal study.

To produce enough material for further animal studies and to investigate extract procedures at pilot/production scale we sourced *A. nodosum* powder from a commercial supplier (Acadian Seaplants Ltd., Dartmouth, N.S.). With this material we produced polyphenol extract (PPE), an enriched polyphenol fraction (PPE-F1) and a polysaccharide-enriched extract (PSE). The polyphenol contents of PPE and PPE-F1 were 29.5% and 65.7%, respectively, whereas their IC_{50} values for α -glucosidase inhibition were 84.8 μ g/mL and 27.2 μ g/mL, respectively. Thus PPE and PPE-F1 are comparable in polyphenol content and α -glucosidase inhibitory activity to ON-E1 and ON-F2 (see Table 1). In addition to polyphenolic content and α -glucosidase inhibition activity, we used proton NMR to assess the polyphenolic profile of extract fractions. This showed that various batches of product produced at pilot scale were consistent in terms of polyphenolic constituent profile (results not shown). Thus PPE and PPE-F1 used in the present studies have a polyphenolic profile consistent with previous batches. In addition, storage of PPE-F1 (at 4 °C) for up to 2.5 years, did not appreciably alter phenolic peaks (results not shown) suggesting the polyphenolic constituents and their bioactivity are stable.

STZ-diabetic mice were treated for up to 4 weeks by daily gavage with a PPE, PPE-F1 or PSE. For comparison, one group of animals received the antidiabetic drug metformin. Table 2 outlines the various groups and the effect of treatments on body mass. Animals made STZ-diabetic

Fig. 1. Uptake of 2-deoxyglucose by 3T3-L1 adipocyte cells incubated with ON-E1. Differentiated 3T3-L1 cells were incubated overnight in DMEM containing 0.2% FBS and treated for 20 min without or with insulin (5 nmol/L) and containing, or not, various concentrations of the *A. nodosum* aqueous ethanolic extract (ON-E1). Cells were then incubated in uptake buffer containing [³H]deoxyglucose for 10 min. Results are for a single experiment with triplicate determinations.



became visibly thinner and were less active. During the 4-week period, nondiabetic mice gained approximately 19 g in body mass. This increase did not occur in STZ-diabetic mice. None of the *A. nodosum* treatments or metformin improved this situation (Table 2). STZ-diabetic mice had increased food and water intake compared with nondiabetic mice, but there were no differences in food and water consumption among the various treatment groups (data not shown).

Blood glucose levels were elevated in STZ-treated mice (Table 3). Metformin treatment lowered serum glucose, but this effect reached statistical significance only on days 14 and 28 of the study; moreover, glycemia remained elevated compared with that of nondiabetic controls. Similarly, PPE and PPE-F1 resulted in a significant reduction in fasting glucose level on day 14, but the reduction was not statistically significant at the other time points. Although fasting serum glucose tended to be lower in the PSE group than in the STZ-diabetic group, this did not reach statistical significance at any of the time points tested.

Total serum cholesterol tended to be elevated in STZ-diabetic mice compared with nondiabetic controls (2.67 ± 0.12 mmol/L versus 2.16 ± 0.15 mmol/L) although not significantly so (Table 4). Treatment of STZ-diabetic mice with metformin, PPE, or PPE-F1 lead to significant reductions ($p < 0.05$) in cholesterol level to 1.93 ± 0.22 mmol/L, 1.99 ± 0.24 mmol/L, and 2.00 ± 0.14 mmol/L, respectively. Glycated serum protein was also elevated in STZ-diabetic mice compared with the nondiabetic control group (6.20 ± 0.18 mmol/L versus 4.94 ± 0.14 mmol/L, $p < 0.05$; Table 4). Metformin, PPE and PPE-F1 reduced glycated serum protein level to 5.22 ± 0.17 mmol/L, 5.15 ± 0.14 mmol/L and 5.47 ± 0.19 mmol/L ($p < 0.05$ compared with the STZ-diabetic group), respectively. PSE treatment was without effect.

Glycogen level in the liver was decreased in STZ-diabetic mice compared with the level in nondiabetic mice ($4.41 \pm$

0.92 mg/g wet mass of liver tissue versus 6.70 ± 1.08 mg/g wet mass of liver tissue, $p < 0.05$; Table 4). PPE treatment normalized liver glycogen (6.49 ± 1.6 mg/g wet mass of liver tissue, $p < 0.05$), whereas metformin, PSE, and PPE-F1 treatments were without significant effect. Interestingly, metformin, PPE, PSE, and PPE-F1 treatments lead to significant elevation in the antioxidant capacity of serum (Table 4).

Both PPE and PSE mildly elevated liver ALP, an enzyme marker of liver damage. However, liver tissue from PPE and PSE-treated mice appeared normal under histological examination (results not shown).

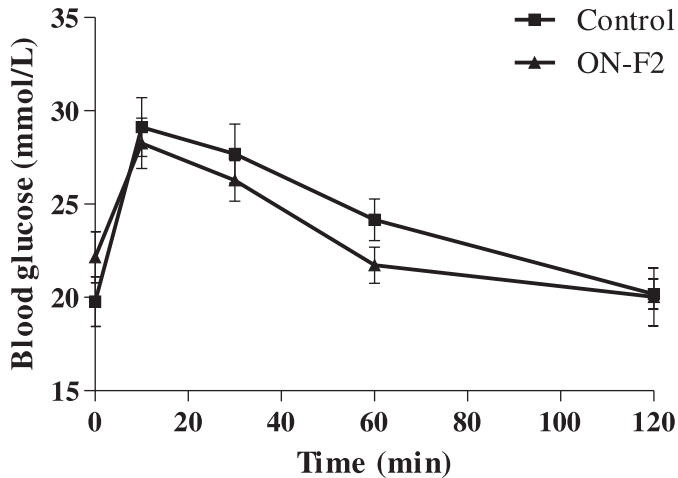
To further examine the potential role of the α -glucosidase inhibitory activity of PPE-F1 in vivo, an oral sucrose challenge test was performed on STZ-diabetic mice (Fig. 3). Acarbose, a pharmaceutical α -glucosidase inhibitor, markedly reduced glycemia after a sucrose challenge. PPE-F1 treatment produced a milder effect that was statistically significant at 10 min after the sucrose challenge.

Discussion

Ascophyllum nodosum, an abundant seaweed of Atlantic Canada, is rich in minerals and nutrients. It is commercially valuable as a feed supplement for agricultural animals and as a fertilizer ingredient for soil amendment, as well as having a history of use as a human foodstuff. Previously, we reported that *A. nodosum* is a rich source of polyphenols and has significant antioxidant scavenging activity toward the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical (Zhang et al. 2006). *A. nodosum* and other brown seaweeds contain phlorotannins (compounds that have phloroglucinol as structural units and have a molecular size from tens of thousands to a million Da for polyphloroglucinols). Initial chemical characterization of the polyphenolics using NMR suggests that phlorotannins structures of high molecular mass are responsible for the α -glucosidase inhibitory activity (results not shown). In addition, structural analysis did not uncover the presence of any brominated compounds so the polyphenols are distinct from the α -glucosidase-inhibiting bromophenols of red seaweed reported by (Kurihara et al. 1999).

Polyphenols are one of the major categories of natural products that are important to human health (Shahidi and Naczki 2004). There is a growing literature on the benefit of adequate amounts of polyphenolic compounds in the diet and prevention of degenerative diseases such as cancer, cardiovascular diseases, and diabetes (Manach et al. 2005; Scalbert et al. 2005). It is generally believed that dietary polyphenolic compounds are protective against the oxidative stresses associated with disease states, thereby delaying onset or slowing disease progression. It is clear from our data that the α -glucosidase inhibitor activity of PPE and PPE-F1 is not the sole reason for the antidiabetic properties observed here and that antioxidant properties and (or) other bioactivities also play a role. This may be why PPE was at least as effective as PPE-F1, or more so in some cases, when administered to STZ-diabetic mice. Indeed, effects on antioxidant capacity in blood, cholesterol-lowering, and reduction in glycated serum protein argue for additional mechanisms. The effect of PPE and PPE-F1 on blood glucose was only evident at day 14 (Table 2) comparable to metformin which

Fig. 2. The effect of a polyphenol-enriched fraction (ON-F2) on blood glucose during a maltose challenge in type II diabetic mice (C57BL/6J strain). Mice were made diabetic by a combination of diet (high-fat and high-sucrose feeding) and injections of low-dose STZ (35 mg/kg body mass). Mice were deprived of food for 2 h and then gavaged with ON-F2 (300 mg/kg body mass; triangles) or 0.25 mL PBS (controls, squares). After 5–10 min, mice were gavaged with 2 g of maltose/kg body mass. A drop of blood was taken from the saphenous vein at the times indicated. Results are means \pm SE of 7 animals per group. There was no significant effect of treatment at any of the time points (two-way repeated measures ANOVA).

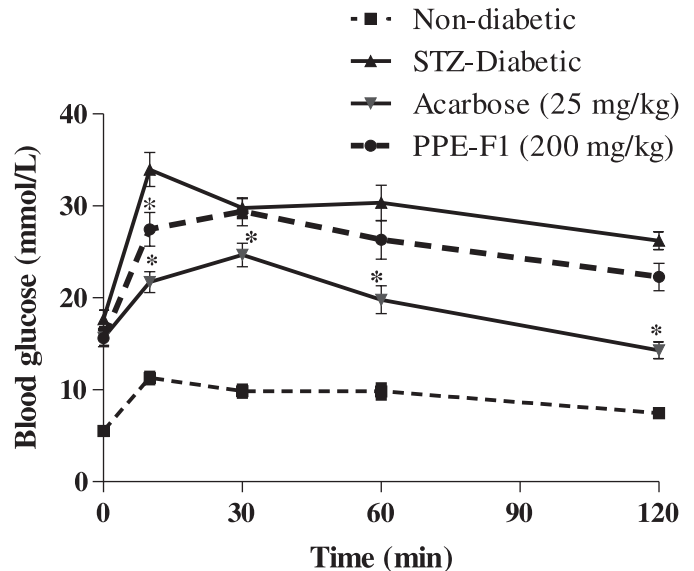


lowered glycemia on days 14 and 28. An increase in blood glucose was observed in the STZ-diabetic mice on day 28. Blood was taken from the tail vein between 09:00 and 10:00, in a manner consistent with the other time points, so we do not believe that blood collection method was a factor. Also, the increase in blood glucose in the STZ-diabetic mice was not evident in the metformin treatment group, suggesting that this was a real effect that was prevented by metformin. It may be that the diabetic state in these animals was not stable and that there was a worsening of glycemia at 4 weeks.

A. nodosum is also a good source of sulphated polysaccharides, compounds that also are reported to have various biological activities including effects on blood coagulation and hyperlipidemia (Liu et al. 2002; Yao et al. 2006). Our study here showed that PSE, a sulphated polysaccharide-enriched extract, had little effect on the parameters measured except for augmenting antioxidant capacity of the serum.

The beneficial effects produced by PPE in STZ-diabetic mice compare well with those of metformin (dimethylbiguanide), a frequently prescribed antidiabetic drug that lowers blood glucose levels by decreasing hepatic glucose output and stimulating glucose disposal in skeletal muscle. Recent work has shown that metformin stimulates AMP-activated protein kinase (AMPK), which leads to stimulation of glucose uptake and fatty acid oxidation by muscle and inhibition of glucose production in the liver (Zhou et al. 2001). Interestingly, polyphenol compounds including resveratrol, the major polyphenol of red wine, have been shown recently to also stimulate AMPK (Zang et al. 2006) and resulted in a decreased lipid accumulation in liver of diabetic mouse. It

Fig. 3. The effect of a polyphenolic-enriched fraction from *A. nodosum* on sucrose tolerance in diabetic mice. Mice (Kunming strain) were made diabetic with a single injection of STZ (110 mg/kg body mass). They were fasted overnight and then gavaged with a single dose of a polyphenolic-enriched fraction (PPE-F1; 200 mg/kg body mass; circles), acarbose (25 mg/kg body mass; inverted triangles), or saline (STZ-diabetic control group; triangles). For comparison, a group of nondiabetic animals were gavaged with saline (squares). Ten minutes after gavaging of samples, blood was sampled from the tail vein for glucose measurement (time 0). The animals were then gavaged with sucrose (2 g/kg body mass). Blood was sampled from the tail vein at 10, 30, 60, and 120 min after the sucrose challenge for glucose measurement. Data are means \pm SE of 10 animals per group. *, significant difference compared with value at the same time point for the STZ-diabetic group ($p < 0.05$, Bonferroni multiple comparison test).



would be of interest to determine whether the polyphenolic compounds from *A. nodosum* stimulate AMPK and whether this contributes to their antidiabetic activity.

The goal of this research is to develop nutraceutical supplements and functional food ingredients that could be used in the maintenance of healthy blood glucose levels. The polyphenolic constituents of *A. nodosum* exhibit promising antidiabetic characteristics and are amenable for use as food supplement ingredients. The use of such a nutraceutical or food supplement could be part of a strategy involving lifestyle change that has been shown in the Diabetes Prevention Program to reduce the risk of diabetes in glucose-intolerant individuals (Diabetes Prevention Program Research Group 2002). To develop such a product, more work will be needed to better understand the mode(s) of action, bioavailability, and safety of ingesting polyphenols in concentrated forms.

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