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Polysaccharides from the green alga *Ulva rigida* improve the antioxidant status and prevent fatty streak lesions in the high cholesterol fed hamster, an animal model of nutritionally-induced atherosclerosis

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ABSTRACT

We investigated the effects of *Ulva* in hypercholesterolemic hamsters, before and after processing. Three groups of 12 hamsters were fed a high cholesterol diet for 12 wk (Control) or a high cholesterol diet where cellulose has been replaced for an equivalent fibre weight from *Ulva* or processed *Ulva*. Plasma cholesterol, non-HDL cholesterol and specially triglycerides were reduced by *Ulva*. Liver glutathione peroxidase activity was increased and thiobarbituric acid reactive substances were efficiently reduced by dietary treatments compared with controls, whereas plasma antioxidant capacity was increased and aortic fatty streak area was decreased by 70%. The results show for the first time that chronic consumption of polysaccharides supplied by *Ulva* prevent the fall of antioxidant defences and the development of atherosclerosis in hamsters. The underlying mechanism is related mainly to increased antioxidant status although improvement of the serum lipid profile was not ruled out.

1. Introduction

The postulated involvement of lipid peroxidation in atherogenesis invoked intensive research on antioxidants. Many compounds, from natural sources, newly synthesised derivatives of these compounds, and common drugs, were tested for their potency to inhibit the peroxidation of plasma lipids induced by different oxidants in vitro (Rice-Evans, Leake, Bruckdorfer, & Diplock, 1996) to reduce the plaque formation in animal models *in vivo* (Barwicz, Gruda, & Tancrede, 2000) and to reduce the morbidity and mortality in human subjects, in clinical trials (Aviram et al., 2000). Indeed, mortality from cardiovascular disease is the leading cause of death in the industrialised world. Diet is believed to play a major role in the development of this disease, and much research is being focused on identifying ways to prevent it through changes in dietary habits. Oxidation of low-density lipoproteins (LDL) is traditionally accepted as initiating processes leading to the development of atherosclerosis. The earliest events in the development of the pathology are endothelial dysfunction and oxidative stress in the vascular cell wall, activation of inflammatory cells, and migration of vascular smooth muscle cells to the intima with the modification of the extracellular matrix, leading to the artery remodelling. Development of atherosclerosis is thought to be closely dependent upon increased oxidative stress, that is, an imbalance between reactive oxygen species (ROS) generation (chiefly superoxide anions, hydrogen peroxide, hydroxyl radicals) and natural cell antioxidant capacity in favour of the former (Frei, 1994). ROS can also regulate many signalling pathways, such as infiltration of monocytes in intima and vascular smooth muscle cell proliferation. A potentially important clinical corollary of the atherosclerosis oxidation theory is that inhibition of LDL oxidation may also inhibit atherosclerosis independent of lowering plasma cholesterol concentrations. Thus, it is essential to develop and utilise effective and natural antioxidants so that they can protect the human body from free radicals and retard the progress of many chronic diseases. Published data indicates that plant polysaccharides in general have antioxidant activities and can be explored as novel potential antioxidants (Ng et al., 2004; Jiang, Jiang, Wang, & Hu, 2005; Wang & Luo, 2007). Different polysaccharide fractions extracted and purified from litchi fruit pericarp tissues exhibited strong antioxidant activities (Yang et al., 2006). Tea polysaccharides were also shown to exert significant inhibitory effects on hydroxyl and superoxide radicals and lipid peroxidation. They could also improve the activity of superoxide dismutase (SOD). These results suggested that tea polysaccharides were potent antioxidants

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in mice (Chen, Zhang, & Xie, 2005). In addition, polysaccharides extracted from mushrooms, such as Auricularia auricular, have also shown antioxidant properties as shown by their free radical scavenging ability (Fan, Zhang, Yu, & Ma, 2007). The structure and mechanisms of the pharmaceutical effects of bioactive polysaccharides on diseases have been extensively studied, and more natural polysaccharides with different curative effects have been tested and even applied in therapies. Macroalgae have also received much attention as potential natural antioxidants (Duan, Zhang, Li, & Wang, 2006).

Thus, the antioxidant activity of natural polysaccharides from the green alga *Ulva pertusa* was determined *in vitro*, including scavenging activity against superoxide and hydroxyl radicals, reducing power, and chelating ability. They showed strong scavenging activity against hydroxyl radical and chelating ability (Qi et al., 2006).

Golden Syrian hamsters fed a fat-rich diet develop dyslipidemia and atherosclerotic plaques, similar in many respects to human atheroma (Auger et al., 2002; Auger et al., 2005). Hamsters were selected for this study because of their responsiveness to plasma cholesterol lowering and anti-atherogenic interventions. Moreover hamsters have similar plasma lipoprotein distribution to humans and LDL as the major plasma cholesterol carrier. In order to induce an oxidative stress, their high cholesterol and high fat diet was rendered deficient in vitamin C and E and in selenium. This study was designed to trigger the arterial wall response to such a stress (fatty streak formation and aortic atherosclerosis emergence) and then to look at the modulation of this effect by algal polysaccharides. In addition, the modulation of an oxidative stress parameter such as cardiac production of superoxide anions was measured in this model.

2. Material and methods

2.1. Algal extracts

The green algae, *U. rigida*, were collected on the coast of a local lagoon, thoroughly washed with distilled water, freeze-dried and ground. They were extracted with distilled water for 60 min at 90 °C, centrifuged at 4500*g* for 10 min and the supernatant was discarded. The extraction was repeated and the residue, referred to as processed *Ulva* in this paper, was freeze-dried. Total, insoluble and soluble dietary fibre was measured using the method of Prosky, Asp, Scheizer, DeVries, and Furda (1998). Crude algae contained 40% total dietary fibres (21% insoluble, 19% soluble) and processed *Ulva* contained 38.7% total dietary fibres (28.8% insoluble, 9.9% soluble).

2.2. Animals, diets and experimental design

Thirty six male golden Syrian hamsters (Janvier, Le Genest-St-Isle, France) four weeks old and weighing ≈80 g were randomly divided into groups with approximately equal mean group body weights. The animals were housed in polycarbonate cages in a temperature controlled room (23 ± 1 °C) subjected to a 12-h light/dark cycle (lights on at 0700 h) with free access to both food and water. Food intake was recorded daily and body weight every other day. After a 4-day adaptation period, the hamsters were randomly divided into three groups of twelve animals each and fed an atherogenic diet for 12 weeks (Control) consisting of 200 g/kg casein, 3 g/kg L-methionine, 393 g/kg corn starch, 154 g/kg sucrose, 50 g/kg cellulose, 150 g/kg lard, 5 g/kg cholesterol, mineral mix (35 g/kg) and vitamin mix (10 mg/kg). Vitamin and mineral mixes were formulated according to AIN-93 guidelines (Reeves, Nielsen, & Fahey, 1993) and supplied by Scientific Animal Food and Engineering (SAFE, Augy, France); mixes did not contain selenium, vitamin C and vitamin E. The experimental groups were fed the same diet in which cellulose was replaced with an equivalent fibre weight from *Ulva* or processed *Ulva*. Hamsters were handled according to the guidelines of the Committee on Animal Care at the University of Montpellier and NIH guidelines (National Research National Research Council, 1985). At the end of the experimental period, the hamsters were deprived of food overnight, and blood samples were collected under anaesthesia (Pentobarbital) by cardiac puncture. Plasma was prepared by centrifugation at 2,000*g* for 10 min at 4 °C, and then stored at −80 °C until analysis. Plasma total cholesterol (TC) and HDL cholesterol (HDL-C) were determined by commercially available enzymatic methods (respectively nos. CH 200 and CH 203, Randox Laboratories LTD, Crumlin, UK) on a Pentra 400 automated analyser (HORIBA ABX, Montpellier, France). Plasma very low- + low-density lipoprotein cholesterol (referred to as «non-HDL-C in the data tables) was precipitated with phosphotungstate reagent and HDL-C was measured in the supernatant. Plasma triglycerides (TG) were also measured using an automat Pentra 400 and a Randox enzymatic kit (no. TR 1697).The antioxidant capacity of plasma (PAC) was assayed with a quantitative colourimetric technique according to the supplier's instructions (Kit NX2332; Randox, Mauguio, France) and expressed as Trolox equivalent. The assay is based on the incubation of a peroxidase and H_2O_2 with 2,2′-azino-di-(3-ethylbenzthiazoline sulphonate) (ABTS) to produce the radical cation $ABTS^{+}$. This has a relatively stable blue-green colour, which is measured at 600 nm.

2.3. Analytical procedures

The liver was perfused with 0.15 mol/L KCl to remove residual blood, rapidly excised, rinsed in ice cold saline, blotted dry, weighed, sectioned for analyses and stored in liquid nitrogen. Liver was homogenised in 4 volumes of ice cold 0.1 mol/L potassium phosphate buffer (pH 7.4) and the homogenate was spun at 13,000*g* for 15 min at 4 °C. The supernatant was then stored at −80 °C for the subsequent assay of glutathione peroxidase (GSHPx) and superoxide dismutase (SOD) activity on a Pentra 400 analyser. GSHPx activity was measured by the method of Randox (Randox Laboratories LTD, Crumlin, UK) using a commercial kit (Ransel, no. RS505). Superoxide dismutase (SOD) activity was determined using a Randox kit (Ransod, no. SD 125). Lipid peroxidation was determined on the homogenate by measuring the formation of malondialdehyde (MDA), a thiobarbituric acid reactive substances (TBARS), which served as the index of lipid peroxidation. Homogenate (1 mL) was mixed with 17.5% trichloroacetic acid (1 mL) and 0.6% 2-thiobarbituric acid (1 mL). This mixture was incubated one hour at 80 °C and then 70% trichloraoacetic acid (1 mL) was added. After centrifugation at 1000*g* for 15 min, the absorbance of the supernatant was measured at 535 nm. TBARS concentration was expressed as ng/mg protein. Thiobarbituric acid 1,1,3,3-tetraethoxy-propane was used as standard of TBARS (Décordé, Teissèdre, Auger, Cristol, & Rouanet, 2008). Protein content was determined according to Smith et al. (1985) and using bovine serum albumin as a standard.

Following blood collection and liver removal, the intact aorta was first perfused with phosphate buffered saline containing 1 mmol/L $CaCl_2$ and 15 mmol/L glucose for 5 min, then with 0.1 mmol/L sodium cacodylate buffer pH 7.4 containing 2.5 mmol/L $CaCl_2$, 2.5% paraformaldehyde and 1.5% glutaraldehyde for the fixation of the vasculature. The aortic tissue was obtained and processed for fatty streak analysis as previously described (Auger et al., 2002). A computerised image analysis system (Image J, Scion Corporation, Frederick, MD) attached to a light microscope was used to measure the total Oil Red O stained area of each aortic arch. The area covered by macrophage foam cells (aortic fatty streak lesion area or AFSA) highly localised in the aortic arch

region, was expressed as a percentage of the total area surveyed. To determine the superoxide anion production, the left ventricle (Delbosc et al., 2005) was washed three times in Krebs buffer and immediately homogenised and centrifuged at 4,000 rpm for 20 min. The supernatant was used to study NAD(P)H-dependent superoxide production. Lucigenin (10 μM)-enhanced chemiluminescence was used to measure superoxide production with NADPH (100 μM). The intensity of luminescence was recorded on a luminometer (Perkin Elmer Wallac, Victor, Turku, Finland). Results were expressed as count/mg of protein.

2.4. Statistical analyses

Data are shown as the means ± SEM of fourteen measurements per group. Data were subjected to logarithmic transformation when necessary to achieve homogeneity of variances. Statistical analysis of data was performed by one-way ANOVA followed by Fisher's Protected Least Significant Difference post-hoc procedure using a Stat View IV software (Abacus Concepts, Berkeley, CA). Differences were considered to be significant at $P < 0.05$.

3. Results

There was a significant increase in food intake by *Ulva* (12%) and processed *Ulva* (20%). Then body weight gain increased by feeding *Ulva* (67%) and processed *Ulva* (119%) (Table 1). Table 2 details the plasma lipid profile for hamsters. TC levels were significantly lowered in groups fed *Ulva* (14%) compared to controls; this decrease was attributed almost solely to markedly reduced level of non-HDL-C (37%). Processed *Ulva* did not modify plasma cholesterol. Triglycerides were efficiently lowered by *Ulva* (49%) and to a lesser extent by processed *Ulva* (33%). PAC values are also displayed in Table 2; *Ulva* and processed *Ulva* significantly prevented the weak PAC induced by the atherogenic diet compared to a standard diet (1.24 ± 0.09 mmol/L, data not shown here). Antioxidant enzyme activities of liver tissue are summarised in Table 2. SOD activity was not modified by dietary treatments; GSHPx activity significantly increased when hamsters received *Ulva* (53%) or processed *Ulva* (67%). The hepatic TBARS concentration was also shown in Table 2 and was strongly lowered by the consumption of *Ulva* (87%) or processed *Ulva* (86%). After 12 weeks, *Ulva* significantly decreased cardiac $O_2^{\cdot-}$ production by 41% and processed *Ulva* by 31% (Fig. 1). Average AFSA, measured as the percentage of Oil Red O staining relative to the total area surveyed (Fig. 2), was significantly decreased in hamsters receiving *Ulva* (70%) and processed *Ulva* (72%) as compared to controls, according to the same pattern observed for TBARS.

Table 2
Effects of ingestion of *Ulva* and processed *Ulva* on plasma lipid concentrations and plasma antioxidant capacity (PAC) and on hepatic antioxidant enzyme activities and thiobarbituric acid reactive substances (TBARS) in hamsters fed an atherogenic diet for 12 weeks.

	Control	*Ulva* (mmol/L)	Processed *Ulva*
Plasma			
Total cholesterol	7.77 ± 0.31[a]	6.65 ± 0.39[b]	7.30 ± 0.19[ab]
HDL-cholesterol	5.18 ± 0.07[a]	5.04 ± 0.12[a]	4.65 ± 0.14[a]
Non HDL-cholesterol	2.59 ± 0.21[a]	1.63 ± 017[b]	2.65 ± 0.17[a]
Triglycerides	1.76 ± 012[a]	0.89 ± 0.10[b]	1.17 ± 0.07[c]
PAC	0.81 ± 0.05[a]	1.35 ± 0.05[b]	1.28 ± 0.04[b]
Liver			
SOD[A] (U/mg protein)	22.68 ± 1.41[a]	21.32 ± 0.91[a]	22.40 ± 1.04[a]
GSHPx[B] (U/mg protein)	0.43 ± 0.03[a]	0.69 ± 0.13[b]	0.72 ± 0.05[b]
TBARS (ng/mg protein)	27.05 ± 3.27[a]	3.34 ± 0.29[b]	3.87 ± 0.61[c]

Values are means ± SEM, $n = 12$. Means in a row with superscripts without a common letter differ, $P < 0.05$.
[A] SOD: superoxide dismutase.
[B] GSHPx: glutathione peroxidase.

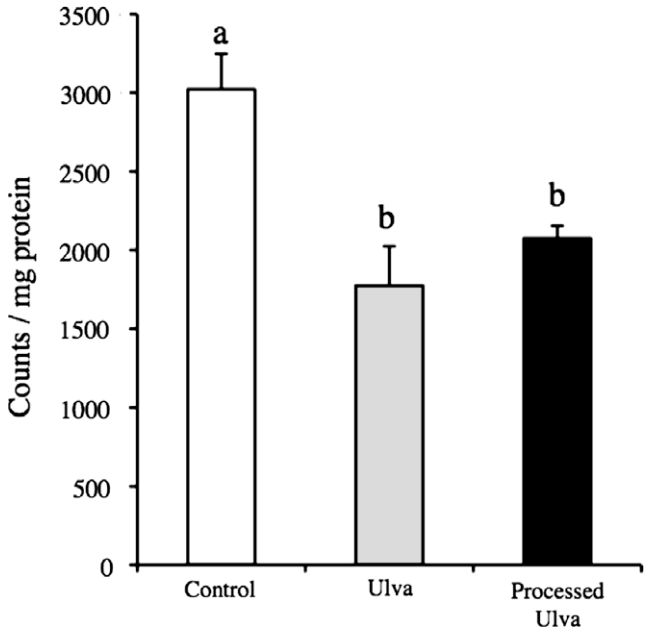

Fig. 1. Cardiac superoxide anion production in hamsters fed high cholesterol diet (control) or a high cholesterol diet where cellulose has been replaced for an equivalent fibre weight from *Ulva* or processed *Ulva* for 12 weeks. Values are expressed as mean ± SEM of triplicate wells ($n = 6$). For each dietary treatment, bars with different index letters differ ($P < 0.05$).

4. Discussion

The current study was designed to examine the cholesterol lowering, antioxidative and antiatherosclerotic activity and possible mechanism(s) of green alga *Ulva*. There is abundant data available from *in vitro* studies or in human subjects and animals which demonstrate the antioxidant properties of plant polysaccharides. In this study we have demonstrated for the first time the cardiovascular and oxidative stress protective effect of crude alga supplementation in high cholesterol fed golden Syrian hamsters. It is well established that elevated blood lipids levels constitute the major risk factor for atherosclerosis (Castelli, 1986). Our results showed that after 12 weeks plasma lipid profiles showed decreased cholesterol

Table 1
Body weight and food intake in hamsters fed a high cholesterol diet (control), or a high cholesterol diet where cellulose has been replaced for an equivalent fibre weight from *Ulva* or processed *Ulva* for 12 weeks.

	Food intake (g/day)	Initial body wt. (g)	Final body wt. (g)	Weight gain (g)
Control	4.03 ± 0.32[a]	92.3 ± 5.0[a]	109.4 ± 10.8[a]	17.2 ± 7.1[a]
Ulva	4.54 ± 0.35[b]	92.1 ± 6.1[a]	120.9 ± 10.4[b]	28.8 ± 5.8[b]
Processed *Ulva*	4.86 ± 0.49[c]	94.0 ± 6.2[a]	131.7 ± 19.8[c]	37.7 ± 9.4[c]

Values are means ± SEM, $n = 12$. Data were analysed by one-way ANOVA followed by the least significant difference test. For each dietary treatment, means in a column with the different letters differ, $P < 0.05$.

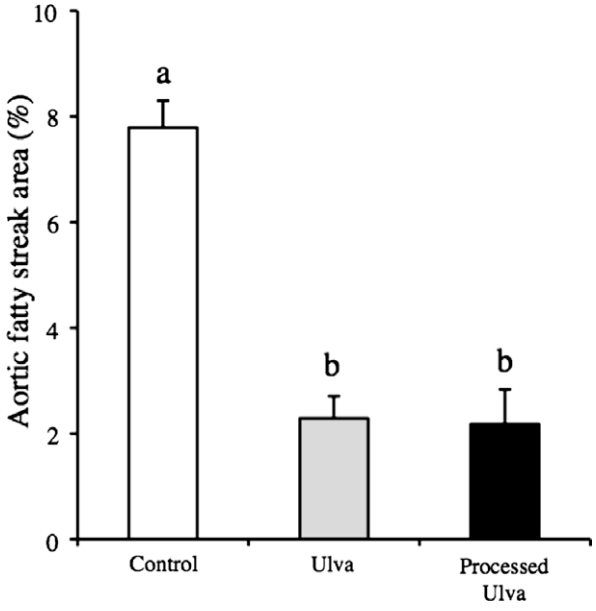

Fig. 2. Effects of feeding a high cholesterol diet (control) or a high cholesterol diet where cellulose has been replaced for an equivalent fibre weight from *Ulva* or processed *Ulva* on aortic fatty streak area in hamsters for 12 weeks. Values are expressed as mean ± SEM ($n = 6$). For each dietary treatment, bars with different index letters differ ($P < 0.05$).

and triglyceride levels on treatment with crude *Ulva*. Hypercholesterolemia plays an important role in atherosclerosis and there is strong evidence that it increases production of ROS and leads to endothelial cell injury, which sets the stage for atherosclerosis (Lusis, 2000). Recent studies have shown that elevated levels of serum triglycerides (TG) are an independent risk factor for coronary heart disease (Stalenhoef & de Graaf, 2008). The atherogenicity associated with high levels of TG is thought to be due to the atherogenic lipoprotein subclasses commonly associated with hypertriglyceridemia (Hodis, 1999). Processing of *Ulva* led to the loss of about 50% soluble polysaccharides, suggesting that they are strongly liable for the hypolipidemic effects observed. Complexes of free radical scavenging enzymes, such as SOD and GSHPx have evolved to prevent excessive oxidant stress. The development of tissue injury probably depends on the balance of the generation of reactive oxygen species and the tissues antioxidant defence mechanism. SOD is the primary enzyme involved in the dismutation of the superoxide radical to hydrogen peroxide and GSHPx is involved in the splitting of hydrogen and lipid peroxides to water, alcohols and hydrogen. An increase in the activities of the liver antioxidant enzyme GSHPx, observed in the present study by feeding algae might be due to the decrease in free radical levels. When they are present in high concentrations they are able to interact with the enzymes and inactivate them (Pigeolet et al., 1990). Supplementation with algal polysaccharides was found to increase the activity of the antioxidant enzymes. Zhang et al. (2003) have reported an increase in antioxidant status in ageing mice on supplementation with polysaccharide fraction from the alga *Porphyra haitanesis*.

Lipid peroxidation, a degenerative pathway of the membrane components mediated through the free radicals produced in the cell, is a hallmark feature of oxidative stress. In the present study, there is an upsurge in lipid peroxidation in liver due to dietary conditions and this was further decreased with supplementation of *Ulva* and processed *Ulva*. An abnormal rise in lipid peroxidation was reduced with *Ulva* polysaccharide administration, due to their antioxidant activity, emphasised through *in vitro* experiments (Qi et al., 2006). This is in line with the previous observation where algal polysaccharide supplementation could circumvent the *in vitro* oxidation of linoleic acid (Tannin-Spitz, Bergman, van-Moppes, Grossman, & Arad, 2005) or human kidney lipid peroxidation (Veena, Josephine, Preetha, & Varalakshmi, 2007).

Elsewhere, the development of atherosclerosis is associated with an increased cardiac ROS production (Heymes et al., 2003). Here superoxide anion production induced by the atherogenic diet, is closely related to the overexpression of NAD(P)H oxidase (Riss et al., 2007; Sutra et al., 2007). As suggested by previous studies in rat models (Al-Awwadi et al., 2005), and in humans (Heinloth, Heermeier, Raff, Wanner, & Galle, 2000), we suspected that the source of superoxide anion generation may be NADPH oxidase. Moreover, a high cardiac level of superoxide plays an important role in the pathogenesis of atherosclerosis and linked to coronary artery disease. In our model, the origin of cardiovascular alterations is accompanied by an increase of cardiac superoxide production. We have recently reported such a cardiovascular complication in the cholesterol fed hamster and the implication of oxidative stress in the process of aortic fat deposition (Riss et al., 2007; Sutra et al., 2007). Here, the superoxide anion production induced by the atherogenic diet is prevented by feeding *Ulva* and processed *Ulva* which prevented aortic fat deposits. However, the exact explanation of the mechanism underlying the free radical scavenging activity exerted by polysaccharides is still not fully understood. The decrease of aortic fatty steak area by alga suggests that ROS-scavenging effect is a likely mechanism since the above studies have established that polysaccharides not only scavenge ROS but also act as potent antioxidants and inhibit the lipid peroxidation mediated by ROS. This major problem remains unresolved. It is unlikely that the antioxidant effects of *Ulva* polysaccharides are attributable to their systemic activity because they are not absorbed. What type of antioxidant is responsible for the observed effects and by what mechanism does it act remains a major problem unresolved. At this stage, we can only propose a hypothesis. The most plausible is that these polysaccharides act only within the lumen by protecting the body against the absorption of radical species and/or by protecting algal antioxidants and nutrients against a possible oxidation before their absorption. Also, the antioxidant effect of some metabolites, produced from polysaccharide fermentation by gut flora, cannot be ruled out.

5. Conclusion

To summarise, the supplementation of *Ulva* and processed *Ulva* to atherosclerotic hamsters is effective in decreasing the oxidative stress and atherosclerosis development, by increasing the activities of antioxidant enzymes like SOD and GSHPx, limiting lipid peroxidation and superoxide anion production. Nevertheless, *Ulva* contains also abundant vitamins and trace elements (Lahaye & Jegou, 1993) that could act sinergistically with polysaccharides. Thus, *in vivo* studies need to be further performed with purified polysaccharides.

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