CHITOSAN OR HYDROLYSED CHITIN GLUCAN EFFICIENTLY PROTECT HYPERCHOLESTEROLEMIC HAMSTERS AGAINST AORTIC FATTY STREAK ACCUMULATION

A. Bornet (,**), K. Decorde (***), J.M. Rouanet (***) , P.L. Teissedre(****)*

(*)Université Montpellier 1 ; Centre d'œnologie, UMR 1083 Sciences pour l'œnologie, 15, av. charles flahault, B.P. 14491, 34093 Montpellier cedex5 FRANCE

(**) Kitozyme SA, Parc Industriel des Hauts-Sarts, Zone 2, rue haute claire, 4, 4040 Herstal, BELGIUM

(***)Equipe d'Accueil 3762, Nutrition & Aliment, Université Montpellier 2, Place Eugéne Bataillon, 34095 Montpellier, FRANCE.

(****) Université Victor Ségalen Bordeaux 2 ; Faculté d'œnologie-UMR 1219 œnologie ampélologie-ISVV laboratoire de chimie appliquée 351 rue de la libération 33405 Talence cedex FRANCE

Abstract : The effects of chitosan, hydrolysed chitin-glucan (from Kitozyme production) on early atherosclerosis were studied in hamsters. Hamsters (n=24) were divided into 3 groups of 8 and fed on atherogenic diet for 12 weeks. They received by force feeding 42,85 mg/kg of body.weight/day : chitosan (C) or hydrolysed chitin-glucan (CG) in water (A dose mimicking a consumption of 3g/Day/70kg Adult Body Weight) . Controls (C) received water by force feeding. After 12 weeks of feeding and 18 h of food deprivation, the hamsters were anesthetized with an intraperitoneal injection of pentobarbitural. Blood was drawn by cardiac puncture with heparin-moistened syringes and plasma was analysed. The aortic arches were carefully dissected and lipids stained with Oil red O. Each aortic arch was then directly displayed on a glass slide, endothelium side up and observed en face by light microscopy. All segments were photographed using a video digitizer. The area covered by foam cells (aortic fatty streak area) was analyzed quantitatively using a computer-assisted morphometry system and expressed as a percentage of the total area surveyed.Plasma total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerids, and aortic fatty streack area were studied. In group that consumed chitosan (C) a reduction of 23,5% for plasma cholesterol and 69,4 % for triglycerides. In the group that consumed hydrolysed chitin-glucan (CG) a reduction of 18,4% for plasma cholesterol and 47,0% for triglycerides. High-density lipoproteins cholesterol was significantly highest with spectacular levels: +222,9% for the group that consumed chitosan (C) and +252,6% for the hydrolysed chitin-glucan (CG) group in comparison with controls (C). A spectacular significant decrease in Low-density lipoproteins cholesterol was found : –87,8% for the group that consumed chitosan (C) and –88,7% for the group consuming hydrolysed chitin-glucan (CG) in comparison with controls. Aortic fatty streak area was significantly reduced in groups receiving chitosan C (96%) or hydrolysed chitin-glucan CG (97%) in comparison with the control group.

Introduction

The name “chitin” is derived from the Greek word “chiton” meaning a coat of mail and was apparently first used by Bradconnot in 1811. Chitin is the most abundant natural biopolymer after cellulose. The chemical structure of chitin is similar to that cellulose with 2-acetomido-2-deoxy-β-D-glucose (NAG) monomers attached via β(1→4) linkages.
Chitosan is the deacetylated (to varying degrees) form of the chitin, which, unlike chitin, is soluble in acidic solutions. Chitosan has three types of reactive functional groups, an amino group as well as both primary and secondary hydroxyl groups at the C-2, C-3 and C-6 positions, respectively.
Chitin is the major structural component of the exoskeleton of invertebrates and the cell walls of fungi.
Chitosan has been extensively researched and many industrial, medical and biomedical applications have been identified. In the USA alone over 300 patents based on chitosan have been filed. Medical applications cited include: haemostatic agent, immunostimulant, drug delivery matrix, skin substitute, wound dressing, control of obesity and lowering of serum cholesterol. Chitosan carries a positive charge on the acetyl remnants and, when solubilised in an acid environment, the chitosan polymers bind to negatively charged molecules such as fats and lipids. Chitosan is not hydrolysed by human digestive enzymes and behaves as a dietary fibre.

Material and Methods

Animals. Weanling male Syrian golden hamsters were received from Elevage Janvier (Le Genest-St-Isle, France) weighing 60-80 g and were randomly separated into three groups (n = 8/group) of statistically equal weight. There were maintained in plastic cages in temperature-controlled enviromment (23 ± 1°C) subjected to a 12-h light/dark cycle and allowed free access to both food and water.

Diets and feeding procedures. Hamsters were fed a semipurified atherogenic diet in which the cholesterol content had been set at 0.5% and which was supplemented with 15% lard at the expense of starch and sucrose; no selenium, vitamin C, or vitamin E was added to this diet. Animals were given food daily for 12 weeks, and uneaten food was weiged daily. The hamsters of each group were additionally force feding once a day either trap water (control) or a solution of chitosan (C) or hydrolysed chitin-glucan (CG) in water. The volumes for solutions force-fed were adjusted daily to the weight of hamsters: a dose mimicking a consumption of 3g/Day/70kg Adult Body Weight to the equivalent for the daily weight of hamsters. Hamsters received 42,85 mg of chitosan or hydrolysed chitin-glucan/(kg of body.weight/day).

Analytical procedure. At the end of 12-week experimental period, hamsters were deprived of food for 18 h and were anesthetized with an intraperitoneal injection of pentobarbitural (60 mg/mL at a dosage of 60 mg/kg of body wt). Blood was drawn by cardiac puncture with heparin-moistened syringes and plasma was prepared by centrifugation at 2000g for 10 min at 4°C and then stored at -80°C until analysis.
Plasma total cholesterol (TC) and high-density lipoprotein cholesterol (HDL-C) were determined by commercially available enzymatic methods (respectively no.1489232 and 1930672, Roche Diagnostics, Mannheim, Germany). Plasma very low- and low-density lipoprotein cholesterol (nonHDL-C) were precipitated with phosphotungstate reagent and HDL-C was measured in the supernatant.

Aortic Tissue Processing. Following blood collection and liver removal, the intact aorta was the first perfused with phosphate-buffered saline containing 1 mmol/L $CaCl_2$, 2.5% paraformaldehyde, and 1.5% glutaraldehyde for the fixation of the vasculature. The aorta was carefully dissected

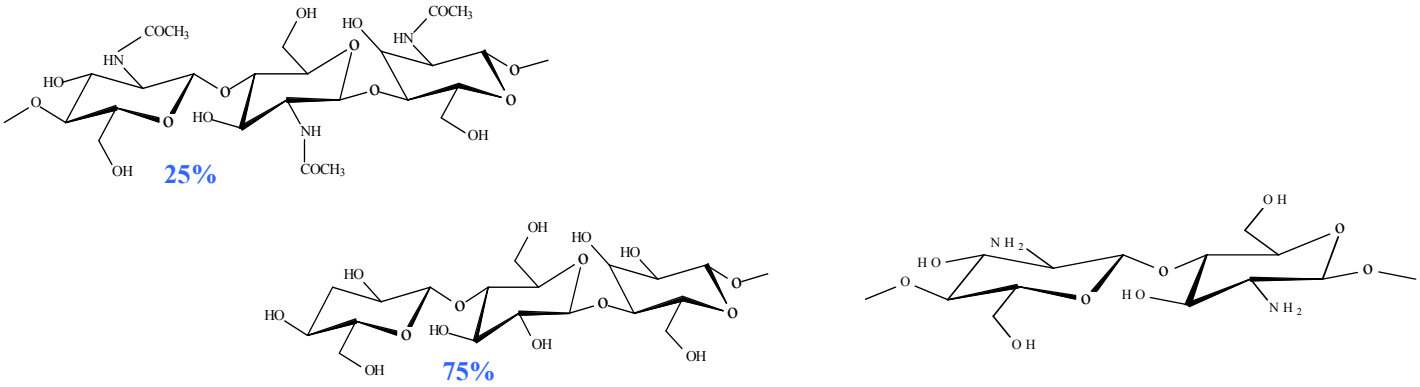
between sigmoid valves and 3-4 cm after the aortic arch and thoroughly cleaned of loose adventitial tissue; the aortic arch was cut free, opened longitudinally along the outside of the arch, pin cork, immersed in fresh fixative solution, and stored at 4°C until staining. The aortic arches were then first rinsed for 48 h in 0.1 mol/L sodium cacodylate buffer (pH 7.4) containing 30 mmol/L $CaCl_2$ and 250 mmol/L sucrose. The arches were then rinsed in distilled water, stained for 40 s in Harris hematoxylin, and then rinsed in distilled water and quickly in 70% isopropyl alcohol; finally, they were stained in Oil Red O for 30 min according to the method of Nunnari and al, rinsed in 70% isopropyl alcohol, and back to distilled water. Each aortic arch was then directly displayed on a glass slide, endothelium side up, covered with Aquamount mounting medium and cover slips and observed an face by light microscopy. All segments were photographed using a video digitizer.The area covered by foam cells (aortic fatty streak lesion) was analyzed quantitatively using a computer-assisted morphometry system and expressed as a percentage of the total area surveyed.

Stastiscal analyses. Data are shown as the means ± SEM, n = 8 measurements/group. Data were subjected to logarithmic transformation when necessary to achieve homogeneity of variances, Stastical analyses of the data were carried out using Stat View IV software (Abacus Concepts, Berkeley, CA) by one-way ANOVA followed by Fisher's protected least significant difference test. Differences were considered to be significant at $P< 0.05$.

Results and Discution

No mortality was observed in any treatment groups. Plasma cholesterol and triglycerides concentrations were significantly reduced. In group that consumed chitosan (C) a reduction of 23,5% for plasma cholesterol and 69,4 % for tiglycerides. In the group that consumed hydrolysed chitin-glucan (CG) a reduction of 18,4% for plasma cholesterol and 47,0% for triglycerides. High-density lipoproteins cholesterol was significantly highest with spectacular levels: +222,9% for the group that consumed chitosan (C) and +252,6% for the hydrolysed chitin-glucan (CG) group in comparison with controls (C). A spectacular significant decrease in Low-density lipoproteins cholesterol was found : –87,8% for the group that consumed chitosan (C) and –88,7% for the group consuming hydrolysed chitin-glucan (CG) in comparison with controls. Average aortic fatty streak accumulation (AFSA), measured as the percentage of Oil Red O staining relative to the total area surveyed was significantly decreased in hamsters receiving chitosan (96%) or hydrolysed chitin-glucan (97%) in comparaison in controls.

Our results demonstrate that an hypercholesterolemic animal model developing atheroslosis treated with chitosan or hydrolysed chitin-glucan (an equivalent dose of 3g for human (70kg)) prevent the development of artherosclerosis through indirect possible mechanisms. Chitosan is a fiber made up of positively charged molecules. When the negatively charged fats and lipids enter the body, they attract with the chitosan and he absorbs the fat so it cannot enter the bloodstream. Chitosan contribute to protective effect against early atherosclerosis. Moreover, in this study, we have demonstrated that chitosan and hydrolysed chitin glucan increase HDL- cholesterol and decrease LDL-cholesterol. Moreover, chitosan and hydroloysed chitin-glucan were particularly efficient in reducing AFSA.



Hydrolysed chitin-glucan (CG)
Short copolymer of chitin and β-glucans

Chitosan (C)
N-acetylated form of chitin

Figure 1 : Stucture of hydrolysed chitin-glucan and chitosan

Diets ingredient	Expti diet
Casein	200
DL-méthionine	3
Cornstarch	393
Sucrose	154
Cellulose	50
Mineral mix[1]	35
Vitamin mix[2]	10
Lard	150
Cholesterol	5

[1]mineral mixture contained (mg/kg of diet): $CaHPO_4$, 17200; KCl, 4000; NaCl, 4000; MgO, 420; $MgSO_4$, 2000; Fe_2O_3, 120; $FeSO_4.7H_2O$, 200; trace elements, 400 ($MnSO_4.H_2O$, 98; $CuSO_4$. $5H_2O$, 20; $ZnSO_4.7H_2O$, 80; $CoSO_4.7H_2O$, 0,16; KI, 0,32; sufficient starch to bring to. 40 g (per kg of diet). .
[2]vitamin mixture contained (mg/kg of diet): rétinol, 12; cholécalciférol, 0.125; thiamin, 40; riboflavin, 30; pantothenic acid, 140; pyridoxine, 20; inositol, 300; cyanocobalamin, 0.1; menadione, 80; nicotinic acid, 200; choline, 2720; folic acid, 10; p-aminobenzoic acid, 100; biotin, 0,6;sufficient sarch to bring to 20 g (per kgof diet)

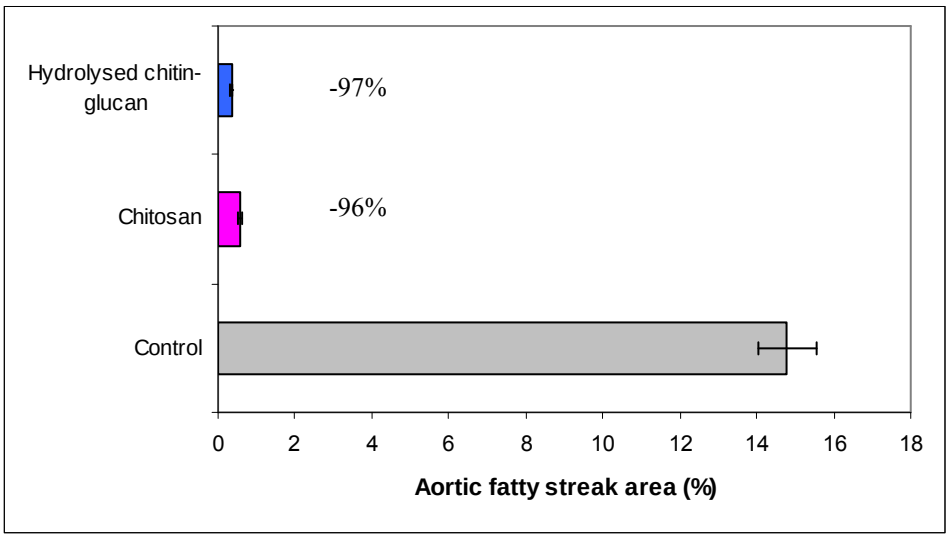
.

Figure 2 : Composistion of diets (g/kg)

Expti group	Control	Chitosan	Hydrolysed chitin-glucan
TC (g/L)	$3,10 \pm 0,30^{a}$	$2,37 \pm 0,03^{b}$	$2,53 \pm 0,21^{b}$
TG (g/L)	$1,96 \pm 0,55^{a}$	$0,60 \pm 0,21^{bc}$	$1,04 \pm 0,27^{bd}$
HDLC (g/L)	$0,57 \pm 0,16^{a}$	$1,84 \pm 0,09^{bc}$	$2,01 \pm 0,11^{bd}$
LDL (g/L)	$2,13 \pm 0,2^{a}$	0,26 ± 0,09b	$0,24 \pm 0,05^{b}$

Values are means ± SEM, n = 8. Data were analysed by one-way ANOVA followed by the least sigificant difference test. For each dietary treatment, means in a column with the different letters differ, P<0.05 Total cholesterol, High-density lipoprotein, triglycerids, low-density lipoprotein.

Figure 3 : Effects on Daily Force Feeding of water (Control), Chitosan and hydrolysed chitin-glucan in water on plasma lipid profile in hamters fed atherogenic diet for 12 weeks.



.
Figure 4 : Effects on Daily Force Feeding of Water (Control), Chitosan, or hydrolysed chitin-glucan in water on aortic fatty streak area (AFSA) in Hamsters Fed Atherogenic Diet for 12 Weeks. AFSA is expressed as a percentage of the total aortic area surveyed

References

[1] Muzzarelli R, Jeuniaux C, Gooday GW, editors. Chitin in Nature and Technology. New York: Plenum Press, 1986.

[2] Muzzarelli RAA. Human enzymatic activities related to the therapeutic administration of chitin derivatives. Cell Mol Life Sci1997;53:131–40.

[3] Subar P, Klokkevold P. Chitosan: the hemostatic agent. Dentistry1992;92:18–22.

[4] Peluso G, Petillo O, Ranieri M, Santin M, Ambrosio L, CalabroD, Avallone B, Balsamo G. Chitosan-mediated stimulation of macrophage function. Biomaterials 1994;15:1215–20.

[5] Otterlei M, Va°rum KM, Ryan L, Espevik T. Characterization of binding and TNF-a-inducing ability of chitosans on monocytes: the involvement of CD14. Vaccine 1994;12:825–32.

[6] Chandy T, Sharma P. Chitosan matrix for oral sustained delivery of ampicillin. Biomaterials 1993;14:939–44.

[7] Bideaux JC, Echinard C, Damour O. Experimental study of a skin substitute. Artificial dermis epidermized with human keratocytes. Chirurgie 1992;118:411–5.

[8] Biagini G, Bertani A, Muzzarelli R, Damadei A, DiBenedetto G, Belligolli A, Riccotti G, Zucchini C, Rizzoli C. Wound management with *N*-carboxybutyl chitosan. Biomaterials 1991;12:281–6.

[9] Ventura P. Lipid lowering activity of chitosan, a new dietary integrator. In: Muzzarelli RAA, editor. Chitin Enzymology, vol. 2. Italy: Atec Edizioni, 1996:55–62.

[10] Veneroni G, Veneroni F, Contos S, Tripodi S, DeBernardi M, Guarino C, Marletta M. Effect of a new chitosan on hyperlipidemia and overweight in obese patients. In: Muzzarelli RAA, editor. Chitin Enzymology, vol. 2. Italy: Atec Edizioni, 1996:63–67.

[11] Maezaki Y, Tsuji K, Nakagawa Y, Kawai Y, Akimoto M, Tsugita T, Takekawa W, Terada A, Hara H, Mitsuoka T. Hypocholesterolemic effect of chitosan in adult males. Biosci Biotech Biochem 1993;57:1439–44.

[12] Weingand KW, Daggy BP, quantitation of high-density lipoprotein cholesterol in plasma from hamsters by differential precipitation. Clin.Chem. 1990; 36,575-580.

[13] Rice-Evans C, Miller NJ, Total antioxidant status in plasma an d body fluids. Methods. Enzymol. 1994; 234, 279-293.

[14] Koechlin C, Couillard A, Cristol JP, Chanez P, Hayot M, Le Gallais D, Prefaut C, Does systemic inflammation trigger local exercise-induced oxidative stress in COPD ? Eur. Respir. J. 2004; 23, 538-544.

[15] Paoletti F., M.A., *Superoxyde dismutase.* Methods in Enzymology., 1990. 186: p. 209-220.

[16] Smith S.K., K.R.I., Mallia A.K., Provenzano M.D., Fujimoto E.K., Goeke N.M., Olson B.J., Klenk D.K.,, *Measurement of protein using bicinchoninic acid.* Anal. Biochem, 1985. 150: p. 76-85

[17] Wendel A. Glutathione Peroxydase. *Methods in enzymology.*, 1981. 77(44), 327-328

[18] Nunnari J. J., Z.T., Joris I., Majno G.,, *Quantification of oil Red O staining of the aorta in hypercholesterolemic rats.* Exp. Mol. Pathol., 1989. 51: p. 1-8.

[19] Hirano S, Akiyama Y. Absence of a hypocholesterolaemic action of chitosan in high-serum-cholesterol rabbits. J Sci Food Agric 1995;69:91–4.

[20] Sugano M, Fujikawa T, Hiratsuji Y, Hasegawa Y. Hypocholesterolemic effects of chitosan in cholesterol-fed rats. Nutr Rep Int 1978;18:531–7.

[21] Sugano M, Watanabe S, Kishi A, Izume M, Ohtakara A. Hypocholesterolemic action of chitosans with different viscosity in rats. Lipids 1988;23:187–91.

[22] Zacour AC, Silva ME, Cecon PR, Bambirra EA, Vieira EC. Effect of dietary chitin on cholesterol absorption and metabolism in rats. J Nutr Sci Vitaminol 1992;38:609–13.

[23] Deuchi K, Kanauchi O, Imasato Y, Kobayashi E. Effect of viscosity or deacetylation degree of chitosan on fecal fat excreted from rats fed on a high fat diet. Biosci Biotech Biochem 1995;59:781–5.