Comparative Study on the Hypocholesterolemic Activity of Amidated Polysaccharides and Psyllium

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The effects of amidated carboxymethylcellulose, amidated pectin, and psyllium on serum and hepatic cholesterol, hepatic fat, and fecal output of sterols were examined in female rats. Rats were fed a diet supplemented with cholesterol (0 or 10 g/kg) and palm fat. Amidated cellulose at 30 g/kg significantly decreased the serum and hepatic concentration of cholesterol by 28.1% and 64.6%, respectively. Corresponding values in rats fed amidated pectin were 28.9% and 72.4%. The effects of psyllium were similar, but less pronounced. Amidated pectin significantly increased the fecal output of cholesterol, total neutral sterols, and total sterols by 49.1%, 31.9%, and 31.0%, respectively. Amidated cellulose and psyllium increased the fecal excretion of total sterols by 1.1% and 5.5%, respectively. In the feces of rats fed amidated cellulose, a small amount of conjugated bile acids was detected (0.83% of total bile acids). In these rats, the lowest expression of hepatic cholesterol 7α-hydroxylase was detected, corresponding to the low fecal output of bile acids. We conclude that the hypocholesterolemic effects of both amidated polysaccharides were similar in spite of their different affinity to sterols.

Keywords: Modified polysaccharides; Dietary sorbents; Sterols; Rats

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INTRODUCTION

Several effective drugs have been developed to treat hypercholesterolemia. Statins are inhibitors of 3-hydroxy-3-methylglutaryl-CoA reductase, which is the rate-limiting enzyme of cholesterol biosynthesis (Farmer and Gotto 1995). Bile acid sequestrants, such as cholestyramine and colestipol, are synthetic resins that bind bile acids in the intestine and increase their fecal excretion. The sequestration of bile acids results in a compensatory increase of bile acid synthesis from cholesterol and decreases the cholesterol concentration in the serum (Nazir *et al*. 1972). Psyllium is a commercially available plant (*Plantago ovata*), that interrupts the enterohepatic circulation of sterols, resulting in their intraluminal entrapment because of the high viscosity of psyllium (Gunness and Gidley 2010).

Hydrophobically modified polysaccharides are sorbents of cholesterol and other neutral sterols and represent an alternative to bile acid sequestrants. Experiments on rats showed that amidated pectin and amidated cellulose are efficient hypocholesterolemic agents (Marounek *et al*. 2013; Tůma *et al*. 2014). In an unpublished pilot-scale experiment by the authors, however, the hypocholesterolemic effects of amidated carboxymethylcellulose and amidated monocarboxycellulose were not consistent with

low fecal excretion of sterols. In rats fed amidated cellulose the fecal output of bile acids was significantly decreased.

The objective of the present study was to compare the hypocholesterolemic activity and fecal output of sterols in rats fed diets containing amidated pectin, amidated cellulose, and psyllium.

EXPERIMENTAL

Materials

The rat diet ST-1 was supplied by Velaz Ltd (Lysolaje, Czech Republic), and protected palm fat AkoFeed Gigant 60 was obtained from AarhusKarlshamn Sweden AB (local branch in Prague, Czech Republic). Psyllium was provided by Dr. Popov, Ltd. (Planá, Czech Republic), and cholesterol, microcrystalline cellulose and carboxymethylcellulose (CMC) were purchased from Sigma-Aldrich (local branch in Prague, Czech Republic). The other chemicals were from Lach-Ner (Neratovice, Czech Republic).

The amidated derivative of CMC with a degree of substitution of 63.1 mol% was prepared using the modified method of Charpentier *et al*. (1997). First, the methylester was prepared *via* esterification with methanol. Then, the methylester was amino-de-alkoxylated with N-octadecylamine. The amidated pectin was N-octadecylpectinamide with a degree of substitution of 60%, prepared *via* the heterogeneous amino-de-alkoxylation of highly methoxylated citrus pectin with N-octadecylamine (Synytsya *et al*. 2004). The degrees of amidation of amidated polysaccharides were derived from their nitrogen and carbon contents. Čopíková *et al.* (2015) presented results of elemental, thermal and spectroscopic analyses (FTIR and NMR), and sorption isotherms of both amidated polysaccharides.

Animals and Diets

Thirty female Wistar rats, approximately six weeks of age, were housed individually in a temperature and humidity controlled room. The animal facility was maintained at a 12 h light: 12 h dark daily photoperiod cycle at a temperature of 22 °C and a relative humidity of 60 ± 5%.

The rats were fed a commercial ST-1 rat diet consisting of soybean meal, meat and bone meal, fishmeal, wheat, maize, oats, wheat bran, limestone, dicalcium phosphate, salt, and supplements of vitamins, trace elements, and amino acids. The rat diet ST-1 contained crude protein, fiber, fat, and cholesterol at 220, 46, 31, and 0.24 g/kg, respectively.

After four weeks, the diet was supplemented with protected palm fat and microcrystalline cellulose at 60 and 30 g/kg, respectively. The rats were randomly divided into five groups of 6 animals each. The average weight of rats at that time was 233 ± 14 g. Experimental diets were supplemented with cholesterol at 10 g/kg at the expense of the palm fat.

Amidated cellulose, amidated pectin, and psyllium were added at 30 g/kg at the expense of the microcrystalline cellulose (Table 1). Food and water were available *ad libitum*. The experiment was approved by the Central Commission for Animal Welfare of the Ministry of Agriculture of the Czech Republic.

Table 1. Composition of Control Diets 1 and 2, and Experimental Diets 3, 4, and 5 Containing Amidated Cellulose (AC), Amidated Pectin (AP), and Psyllium (Psy)

Diet	1[a]	2[a]	3 (AC)	4 (AP)	5 (Psy)
Cholesterol (g)	0	10	10	10	10
Amidated cellulose (g)	0	0	30	0	0
Amidated pectin (g)	0	0	0	30	0
Psyllium (g)	0	0	0	0	30
Palm fat (g)	60	50	50	50	50
Cellulose (g)	30	30	0	0	0
Diet ST-1 (g)	910	910	910	910	910

[a] Control diets without sorbents.

Sampling

Feces were collected during the last five days of the experiment, weighed, pooled, and stored at -40 °C until analysis. The experiment duration was three weeks. At the end of the experiment, the rats received 4 g of feed 4 h before sacrifice (Spielmann *et al*. 2008). Rats were sacrificed *via* decapitation after anesthesia *via* inhalation of isoflurane (Torrex Chiesi CZ, Ltd., Prague, Czech Republic). Mixed blood samples were drawn at the time of sacrifice to obtain the serum. After laparotomy, the livers and ceca were excised, weighed and stored at -40 °C until analysis.

Analyses

The serum levels of total cholesterol, LDL-cholesterol, triacylglycerols, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were determined using commercial kits (Erba-Lachema, Ltd., Brno, Czech Republic). The total hepatic and fecal lipids, fecal neutral sterols, and bile acids were determined as described previously (Tůma *et al*. 2014). Conjugated bile acids in the feces were extracted with methanol for 30 min at 60 °C, applied to a Strata C18-E column (Phenomenex, Torrance, CA, USA), washed with water and acetone, and eluted with methanol (Roda *et al.* 1992). The methanolic solution of bile acid conjugates was analyzed using a Shimadzu (Japan) VP series high-performance liquid chromatograph, with an evaporative light-scattering detector and a Nova-Pak C-18 Waters column. Nordeoxycholic acid was used as the internal standard. Isolithocholic acid, 12-ketolithocholic acid, nordeoxycholic acid, α-, β-, and ω-muricholic acids, and campesterol were purchased from Steraloids Inc. (Newport, RI, USA). Coprostanol, epicoprostanol, cholic acid, lithocholic acid, deoxycholic acid, chenodeoxycholic acid, plant sterols, including campesterol, stigmasterol, β-sitosterol, and β-sitostanol, and conjugated bile acids were purchased from Sigma-Aldrich (Prague, Czech Republic).

RNA Isolation and Real-Time PCR Assay

The RNA isolation and real-time PCR assay were performed as described previously (Marounek *et al*. 2012). Briefly, total RNA was extracted from the liver tissue using an RNeasy Mini Kit (Qiagen, USA), reverse transcribed, and PCR was performed using the QuantiTect SYBR Green RT-PCR Kit (Qiagen, USA). Oligonucleotide primer pairs were used according to Spielmann *et al*. (2008). All data were normalized to the glyceraldehyde-3-phosphate gene in the same sample. The relative mRNA abundance of the cholesterol 7α-hydroxylase gene was calculated using the $2^{-\Delta\Delta C(T)}$ method (Livak and

Schmittgen 2001) and was expressed as the relative values compared to the rats fed the control diet no. 1.

Statistics

Treatment effects were evaluated using a one-way analysis of variance (ANOVA), which was calculated with the Statistica 10 software (StatSoft, Inc., Tulsa, USA). Pooled standard error of the mean (SEM) was calculated using the general linear model procedure within SAS Version 8.2 (Statistical Analytical Systems Institute, Cary, NC, USA). Significant differences ($P < 0.05$) were identified using Tukey's test.

RESULTS

There was no treatment effect on the growth and feed intake of the rats. Their average final weight was 249 ± 16 g. In the first control group, the intake of cholesterol was 10 µmol/day, and in the other groups it varied between 442 and 474 µmol/day. The serum and hepatic cholesterol concentrations in the rats fed the basal diet were 1.97 µmol/mL and 5.19 µmol /g, respectively (Table 2).

Table 2. Effect of Amidated Cellulose (AC), Amidated Pectin (AP) and Psyllium (Psy) on Serum Parameters, Hepatic Cholesterol and Fat, and Liver and Cecum Weights in Rats

Diet	1[a]	2[a]	3 (AC)	4 (AP)	5 (Psy)	Pooled SEM
Serum concentrations						
Total cholesterol	1.97^{bc}	2.60^{b}	1.87^{c}	1.85^{c}	2.27^{bc}	0.14
LDL-cholesterol (µmol/mL)	0^{c}	0.50^{b}	0.06^{c}	0.03^{c}	0.32^{bc}	0.05
Triacylglycerols (µmol/mL)	2.05	1.96	1.72	1.63	1.53	0.27
AST (nkat/mL)	4.04	3.38	3.06	3.26	3.07	0.38
ALT (nkat/mL)	1.20	0.95	1.10	0.93	0.91	0.14
Hepatic concentrations						
Cholesterol (µmol/g)	5.19^{b}	23.40^{c}	8.29^{de}	6.47^{bd}	9.22^{e}	0.12
Fat (mg/g)	70.30^{b}	119.80^{c}	68.90^{b}	73.20^{b}	73.70^{b}	2.45
Weight of liver (g)	9.45	9.31	8.99	9.18	8.40	0.40
Weight of cecum (g)	5.29^{b}	4.92^{b}	5.56^{b}	5.57^{b}	7.43^{c}	0.10

Six female rats per group.
[a] Control diets without sorbents.
Values in the same row with different superscripts differ significantly at $P < 0.05$.

Cholesterol supplementation significantly increased the serum and hepatic cholesterol concentrations to 2.60 µmol/mL and 23.40 µmol/g, respectively. Cholesterol supplementation also significantly increased the LDL cholesterol concentration in the serum from 0 to 0.50 µmol/mL, and the concentration of total lipids in the liver tissue from 70.3 to 119.8 mg/g. In rats fed the diets supplemented with cholesterol, the amidated cellulose and amidated pectin significantly decreased the serum and hepatic cholesterol concentrations, as well as the concentration of total lipids in the hepatic tissue. The corresponding effects of psyllium were similar, but less pronounced. Cholesterol supplementation, amidated polysaccharides, and psyllium did not

significantly influence the activity of the aminotransferases and the weight of the liver. The feces weight, however, was significantly increased in rats fed psyllium.

In all groups of rats fed diets containing cholesterol, the fecal output of cholesterol, neutral sterols, bile acids, and total sterols was significantly increased (Table 3). The amidated pectin significantly increased the fecal output of cholesterol, output of neutral sterols (which is the sum of cholesterol, coprostanol, epicoprostanol, and plant sterols), and output of total sterols (neutral sterols and bile acids). Amidated cellulose and psyllium increased the fecal output of cholesterol, although this was not significant. All three sorbents significantly decreased the output of coprostanol. The feces of rats fed amidated cellulose contained taurocholic acid at 0.151 ± 0.058 µmol/g and taurochenodeoxycholic acid at 0.165 ± 0.096 µmol/g. In the feces of rats of other groups, no conjugated bile acids were present. The fecal output of dry matter and fat was not significantly affected.

Table 3. Effect of Amidated Cellulose (AC), Amidated Pectin (AP) and Psyllium (Psy) on Daily Fecal Output of Dry Matter, Fat, Cholesterol, Neutral Sterols, Bile Acids and Total Sterols in Rats

Diet	1[a]	2[a]	3 (AC)	4 (AP)	5 (Psy)	Pooled SEM
Dry matter (g)	4.01	3.98	3.86	3.89	4.12	0.24
Fat (g)	0.28	0.32	0.36	0.36	0.39	0.07
Cholesterol (µmol)	19[b]	224[c]	247[c]	334[d]	252[c]	13.8
Coprostanol (µmol)	13[b]	62[c]	35[d]	44[d]	40[d]	5.0
Total neutral sterols (µmol)	50[b]	307[c]	314[c]	405[d]	315[c]	14.0
Free bile acids (µmol)	17[b]	41[cd]	34[d]	51[c]	52[c]	4.6
Total bile acids (µmol)	17[b]	41[cd]	38[d]	51[c]	52[c]	2.6
Total sterols (µmol)	67[b]	348[c]	352[c]	456[d]	367[c]	25.2

Six female rats per group.
[a] Control diets without sorbents.
Values in the same row with different superscripts differ significantly at $P < 0.05$.

Table 4. Relative Expression of the Cholesterol Hydroxylase Gene in the Liver Tissue of Rats Fed Control Diets 1 and 2 and Experimental Diets 3, 4, 5 Containing Amidated Cellulose (AC), Amidated Pectin (AP), and Psyllium (Psy)

Diet	ΔC_T[b]	$\Delta\Delta C_T$[c]	$RQ^d(2^{-\Delta\Delta C_T})^d$
1[a]	1.45 ± 0.36	0.00	1.00
2[a]	0.15 ± 0.09	-1.30	2.46*
3 (AC)	1.48 ± 045	0.03	0.98
4 (AP)	0.61 ± 0.39	-0.84	1.79
5 (Psy)	0.07 ± 0.02	-1.38	2.60*

[a] Control diets without sorbents.
[b] The ΔC_T values were determined by subtracting the average GAPDH Ct value from the average 7α-hydroxylase ΔC_T value. The mean ± SD of three measurements for each sample is shown.
[c] Untreated samples (diet no.1) were used as the calibrator. The SD values were the same as those given for the ΔC_T.
[d] The fold change in gene expression normalized to an endogenous reference gene (GAPDH) relative to the untreated control.
* Values differ significantly from control diet 1 at $P < 0.05$

Supplementation of the basal diet with cholesterol significantly increased the relative expression of the cholesterol 7α-hydroxylase gene in the liver tissue (Table 4).

The expression of the cholesterol 7α-hydroxylase gene was also significantly increased in the liver of rats fed psyllium. The lowest expression of the cholesterol 7α-hydroxylase gene was detected in the liver tissue of the rats fed amidated cellulose.

DISCUSSION

Both amidated pectin and amidated cellulose were demonstrated to be efficient hypocholesterolemic agents and were more efficient than psyllium, which is a well-tried food supplement that improves glycemic and lipid control in individuals with mild to moderate hypercholesterolemia (Anderson *et al*. 1999). Amidated pectin and amidated cellulose reduced the serum and hepatic cholesterol and total hepatic lipids to a similar extent. However, their effect on the fecal output of sterols differed. The fecal output of cholesterol, total neutral sterols, and bile acids in rats fed amidated pectin was significantly higher than in rats fed amidated cellulose. The different affinity of amidated cellulose and amidated pectin to sterols was not expected, as both of the modified polysaccharides were octadecylamides with a similar degree of substitution (63.1% and 60.0%, respectively). It can be supposed that the mechanism of lipid sorption by modified polysaccharides is based on hydrophobic interactions of non-polar molecules with long alkyl groups. Due to the residual hydrophilicity, however, amidated cellulose and amidated pectin may also adsorb other molecules.

The lowest fecal concentration and daily output of bile acids were observed in rats fed the diet with amidated cellulose. In these rats, the lowest expression of the hepatic cholesterol 7α-hydroxylase gene was found. Cholesterol 7α-hydroxylase is the rate-limiting enzyme in the synthesis of bile acids. This enzyme activity is enhanced by the treatment of rats with the bile acid sequestrants, cholestyramine and psyllium, as well as by feeding the rats cholesterol (Matheson *et al*. 1995). Rats can convert dietary cholesterol to bile acids (Horton *et al*. 1995), such that they are resistant to hypercholesterolemic diets. The mRNA expression of hepatic cholesterol 7α-hydroxylase was significantly increased in rats fed diet no. 2 containing cholesterol, as well as in rats fed psyllium. Amidated pectin increased the mRNA expression of cholesterol 7α-hydroxylase, although this was not significant. In contrast to other sorbents, in rats fed amidated cellulose, the mRNA expression of hepatic cholesterol 7α-hydroxylase was not increased, which is consistent with the low fecal concentration and output of bile acids. Different biliary bile acid secretion in rats fed cellulose and pectin has been shown by Ide (1994). Dietary cellulose compared to pectin reduced total bile acid secretion to less than one half.

It has been proposed that soluble dietary fibers may lower plasma cholesterol concentration *via* the inhibition of cholesterolgenesis by the propionate produced by the colonic microflora (Chen *et al*. 1984). Amidated polysaccharides and psyllium, however, may decrease the metabolic activity of colonic microflora as shown by lower conversion of cholesterol to coprostanol. Furthermore, previous analyses did not show the increased production of propionate in ceca of rats fed amidated pectin (Marounek *et al*. 2007) or amidated alginate (unpublished results). A more plausible hypothesis is that amidated cellulose may have hindered cholesterol synthesis in the liver and intestine *via* binding to some lipophilic nutrients, which were then excreted in the feces. The side effects of hypocholesterolemic drugs are only partially known. Several reports suggest that cholestyramine affects absorption of the fat-soluble vitamins K and D (Matsui and

Rozovski 1982). Knodel and Talbert (1987) suggested that the adverse effects of cholestyramine may be associated with the impairment of vitamin K and D absorption. Both vitamin K and D participate in various biochemical reactions. Vitamin K is responsible for the carboxylation of glutamate residues in 15 vitamin K dependent proteins (Bandyopadhyay 2008).

Activities of aminotransferases AST and ALT were not increased in rats of experimental groups, thus amidated polysaccharides and psyllium do not negatively influence the hepatocellular health.

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