Low-Methoxyl Pectin Stimulates Small Intestinal Mucin Secretion Irrespective of Goblet Cell Proliferation and Is Characterized by Jejunum Muc2 Upregulation in Rats

Low-methoxyl Pectin Stimulates Small Intestinal Mucin Secretion Irrespective of Goblet Cell Proliferation and Is Characterized by Jejunum *Muc2* Up-regulation in Rats [1, 2]

Shingo Hino[3], Kei Sonoyama[4], Hiroyuki Bito[3], Hirokazu Kawagishi[5], Seiichiro Aoe[6] and Tatsuya Morita[3*]

WORD COUNT: 5953

NUMBER OF FIGURES: 1

NUMBER OF TABLES: 4

SUPPLEMENTARY MATERIAL: 0

RUNNING TITLE: Low-methoxyl pectin stimulates *Muc*2 expression

AUTHOR LIST FOR INDEXING: Hino, Sonoyama, Bito, Kawagishi, Aoe, and Morita

FOOTNOTES

[1] Supported in part by a Grant-in-Aid for Scientific Research from The Ministry of Education, Science, Sports and Culture of Japan.

[2] Author disclosures: S. Hino, K. Sonoyama, H. Bito, H. Kawagishi, S. Aoe and T. Morita, no conflicts of interest.

[3] Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, 836 Ohya, Shizuoka 422-8529, Japan.

[4] Division of Applied Bioscience Graduate School of Agriculture, Hokkaido University, Sapporo 060-8589 Japan.

[5] Graduate School of Science and Technology, Shizuoka University, 836

Ohya, Shizuoka 422-8529, Japan.
[6] Laboratory of Nutritional Biochemistry, Otsuma Women's University, Sanbancho 12, Chiyoda-ku, Tokyo 102-8356, Japan.
[7] Abbreviations used: DMEM, Dulbecco's modified Eagle's minimal essential medium; DE, degree of esterification; GAL, gum arabic of low molecular weight; GGH, guar gum of high molecular weight; GGL, guar gum of low molecular weight; HPC, high-methoxyl pectin; KMH, konjac mannan of high molecular weight; KML, konjac mannan of low molecular weight; LAG, larch arabinogalactan; LPS, lipopolysaccharide; LPC, low-methoxyl pectin; NM, neomycin; SAL, sodium alginate of low molecular weight; SCFA, short-chain fatty acids.

*To whom correspondence should be addressed.
Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, 836 Ohya, Shizuoka 422-8529, Japan.
Tel/Fax: 81-54-238-5132, E-mail: atmorit@ipc.shizuoka.ac.jp

Abstract

Generally, soluble fibers increase small intestinal mucin secretion by increasing the number of goblet cells in a viscosity-dependent manner. The present study aimed to examine the mechanism by which low-methoxy pectin (**LPC**) affects mucin secretion in the small intestine. First, diets containing 50 g/kg of low-viscosity fiber (LPC, gum arabic, guar gum, low-molecular konjac mannan, arabinogalactan, sodium alginate) or high-molecular konjac mannan (**KMH**) were fed to Wistar rats for 10 d. Luminal mucin was greater in the LPC and KMH groups than in the fiber-free control group, but only the KMH group had more goblet cells in the ileum than the others. Next, Sprague-Dawley rats were fed LPC, KMH or high-methoxyl pectin (**HPC**) diets (50 g/kg) for 10 d. The KMH and LPC groups, but not the HPC group, had greater luminal mucin than the control group, while jejunum *Muc2* expression was higher only in the LPC group. Sprague-Dawley rats fed the LPC diet for 1 or 3 d had greater luminal mucin and jejunum *Muc2* expression than those fed the control diet. *In vitro* studies using HT-29MTX cells showed that, of the various fibers studied, only LPC and HPC affected mucin secretion. Finally, Wistar rats were fed the LPC diet with or without neomycin in drinking water for 10 d; neomycin treatment did not compromise the effect of LPC on mucin secretion. We conclude that LPC does not affect the number of goblet cells, but can interact directly with the epithelium and stimulate small intestinal mucin secretion.

Introduction

The mucus layer covering the intestinal mucosal surface acts as a major defensive barrier between the luminal contents and the epithelial cells, protecting them from potential luminal insults (1). This barrier is maintained by intestinal goblet cells that synthesize and secrete mucins, which form the mucus layer (2). Thus, quantitative changes in mucin secretion could have a profound effect on the protective function of this barrier. Indeed, *Muc2*-deficient mice showed increased bacterial adherence to the epithelial cell surface and enhanced susceptibility to colitis (3). In addition, Pullan et al. (4) showed that the colonic mucus layer was thinner in patients with ulcerative colitis than in normal subjects.

Recently, a number of research studies have focused on the interactions of dietary factors with the secretory activity of goblet cells. Dietary intake of threonine (5), certain milk peptides (6), dietary fibers (7, 8) and resistant starch (9, 10) have been shown to be associated with increased mucin secretion in the rat intestine. Shimotoyodome et al. (11) and Barcelo et al. (12) showed that colonic infusion of short-chain fatty acids (**SCFA**), fermentation products of indigestible carbohydrates, stimulated mucin discharge in the rat colon. Additionally, several reports indicate that SCFA, particularly butyrate, increased *Muc* gene expression in the rat colon through an inhibitory effect on histone-deacetylase (13). Toden et al. (9) showed that the thickness of colonic mucus increased in proportion to cecal butyrate concentration in rats fed diets that included graded levels of resistant starch.

There are two types of mucin secretion: “constitutive” (baseline secretion) and “regulated secretion” (2), in which mucin discharge from individual goblet cells is enhanced. Dietary factors such as milk peptides (casomorphine-7) in the small intestine (6, 14) and SCFA in the large bowel have been shown to have effects through accelerated secretion (15). We previously reported that bulky (insoluble) and viscous (soluble) fibers up-regulated baseline secretion of small intestinal mucins by increasing the number of goblet cells in proportion to either the bulk-forming property or the viscosity of the respective fibers ingested (16-19). However, we also found that among the soluble fibers tested, low-methoxyl pectin (**LPC**) was unique in that its mucin-secretory effect was not accompanied by an increase in the number of goblet cells (18). Thus, the mechanism by which LPC stimulates mucin secretion likely differs from that of viscous and bulky fibers. These findings prompted us to hypothesize that LPC molecules would have a direct effect on the epithelial tissue and act as a mucin secretagogue.

Pectin consists mainly of a homogalacturonan backbone and highly substituted rhamnogalacturonan side chain (hairy regions), and galacturonic acid residues are frequently substituted with methyl ester at the C6-carboxyl group (20). In the food industry, pectin is classified into low- (<50%) and high-methoxyl (>50%, **HPC**) pectin based on differences in the degree of esterification of galacturonic acid residues. One study showed that sodium alginate, i.e., mannuronic/guluronic acid-copolymers

secreted by *Pseudomonas aeruginosa*, was a potent mucin secretagogue in isolated ferret trachea (21) and implicated a possible contribution of uronic acid-polymers to the stimulatory effect on mucin secretion. The effects of LPC may also be mediated in this way, since low-methylated galacturonic acid-polymers also possess negative electric charges derived from uronic acids. Another possibility might be related to SCFA produced by microbial fermentation of indigestible carbohydrates, as pectin is rapidly and fully fermented and converted to SCFA when it enters the rat cecum (22). Very active fermentation in the cecum might have an influence even in the lower part of the small intestine; Fukunaga *et al.* reported that increased cecal SCFA and plasma GLP-2 levels following pectin ingestion caused mucosal cell proliferation in the rat ileum (23).

Over 20 years ago, Satchithanandam et al. (24) reported that ingestion of a diet containing 5% citrus fiber (consisting mainly of pectin) increased luminal mucin in the small intestine. However, few studies have examined the mechanism of enhanced mucin secretion following pectin ingestion. The purpose of the present study was to examine the mechanism(s) by which LPC induces mucin secretion in the rat small intestine. We selected several low-viscosity soluble fibers with viscosities similar to or less than that of LPC, and re-examined in greater detail the relationship between fiber viscosity, goblet cell proliferation and luminal mucin content in the rat small intestine. In further experiments, the comparative effects of LPC, a high-viscosity fiber and HPC on intestinal *Muc* gene expression were examined in rats fed the fiber diets for different durations of time. Using

HT-29MTX, a goblet cell line (25), we investigated whether LPC molecules directly stimulate mucin secretion from cells. Finally, to assess the possible contribution of SCFA to the mucin-secretory effect of LPC, neomycin (**NM**) treatment was conducted in rats fed the LPC diet.

Methods

Materials. LPC (Pectin Classic AB901) and HPC (Pectin Classic AM201) of apple origin were gifts from Dainippon Sumitomo Pharm (Osaka, Japan). Konjac mannan of high and low molecular weight (**KMH**; PROPOL A and **KML**; RHEOLEX RS, respectively) was provided by Shimizu Chemical (Hiroshima, Japan). Guar gum of high and low molecular weight (**GGH**; NEOSOFT G and **GGL**; Sunfiber R, respectively) was provided by Taiyo Kagaku (Mie, Japan). Gum arabic of low molecular weight (**GAL**; Gum Arabic SD) and sodium alginate of low molecular weight (**SAL**; KIMICA ALGIN ULV) were obtained from San Ei Gen F.F.I., Inc (Osaka, Japan) and KIMICA Corp. (Tokyo, Japan), respectively. Larch arabinogalactan (**LAG**; Larch AG) was purchased from Vitamin Research Products (Carson City, NV). Dietary fiber content, as determined by the Prosky method (26), was LPC (81%), HPC (88%), KMH (95%), KML (97%), GGH (84.7%), GGL (87%), GAL (81%), SAL (91%), and LAG (~100%) on a dry matter basis. The viscosities of the dietary fiber solutions (1.0 ~ 25% solution, w/v), defined as the area under the viscosity curve described by Dikeman et al. (27), were 2.6 Pa (1% LPC), 8.9 Pa (2% HPC), 599.3 Pa (1% KMH), 34.0 Pa (1% KML), 165.8 Pa (1% GGH), 1.1 Pa (1% GGL), 4.8 Pa (10% GAL), 12.0 Pa (10% SAL), and 3.7 Pa (25% LAG). The degree of

esterification (**DE**) at carboxyl groups of pectin was determined to be 29.5% (LPC) and 58.6% (HPC) by the back-titration method (28).

Care of animals. The study (No. 23-18) was approved by the Animal Use Committee of Shizuoka University, and rats were maintained in accordance with the guidelines for the care and use of laboratory animals of Shizuoka University. Male Wistar (6 wk old) and Sprague-Dawley (4 or 5 wk old) rats were purchased from Shizuoka Laboratory Animal Center (Shizuoka, Japan). The rats were housed individually in screen-bottomed stainless-steel cages in a temperature- (23 ± 2°C) and light- (lights on from 07:00 to 19:00) controlled room. The rats were acclimatized for 5-7 d and fed a control diet (29) formulated from 250 g/kg of casein, 652.5 g/kg of cornstarch and 50 g/kg of corn oil. The remainder of the diet consisted of vitamins including choline (12.5 g/kg) and minerals (35 g/kg). The rats were subsequently allocated to groups based on body weight in order to normalize body weights across the groups. They were allowed free access to experimental diets and water. The experimental fiber diets were formulated by adding dietary fiber to the control diet at the expense of an equal amount of cornstarch. Accordingly, dietary starch levels differed in diets and were 602.5 g/kg for the fiber-added diets. Body weights and food intake were recorded every morning before replenishing the diet.
In *Expt. 1* using Wistar rats (10-d feeding study), we found that the KMH diet resulted in lower food intake and body weight gain than the control diet. Also, even with the LPC diet, lower food intake was observed during the first few days. To avoid a profound effect of lower food intake and

body weight gain on *Muc* gene expression, we used Sprague-Dawley rats in *Expt. 2* (10-d feeding study with KMH and LPC diets) and *Expt. 3* (1- or 3-d feeding study with LPC diet), as our previous findings indicated that Sprague-Dawley rats tolerate soluble fiber-added diet better than Wistar rats.

Expt. 1 After acclimatization for 5 d, 64 Wistar rats, weighing 127-160 g (6 wk old), were divided into 8 groups of 8 rats and allowed free access to the control diet or a diet containing 50 g of LPC, GGL, KML, SAL, GAL, LAG or KMH/kg for 10 d. KMH was used as a positive control based on its effects on goblet cell proliferation and mucin secretion. At the end of the experiment, diets were withdrawn overnight and rats were killed by decapitation, and the small intestine was excised. Luminal contents were collected by flushing the intestine with 15 mL of ice-cold PBS (pH 7.4) containing 0.02 mol/L sodium azide and 20 mL of air. The contents were freeze-dried and stored for luminal mucin analysis. For histologic evaluation, the upper half of the small intestine, excluding the duodenum, was defined as the jejunum and the lower half was defined as the ileum. The mid-portions of the ileum segment were removed and placed in 10% buffered formalin.

Expt. 2 After a 5-d acclimatization period, 64 Sprague-Dawley rats weighing 93-116 g (4 wk old) were divided into 4 groups of 16 rats and allowed free access to the control diet or a diet containing 50 g of LPC, HPC or KMH/ kg for 10 d. Half of the rats in each group were killed by

decapitation without prior food deprivation. The stomach, jejunal and ileal segments (approximately 5 cm) were opened longitudinally, and the mucosa was scraped with a glass slide and used for isolation of total RNA. For the remaining rats, diets were withdrawn overnight and rats were then killed by decapitation. Luminal mucin sampling and collection of intestinal tissue were performed as described for *Expt. 1*.

Expt. 3 After acclimatization for 7 d, 56 Sprague-Dawley rats weighing 174-213 g (5 wk old), were divided into 4 groups of 14 rats and allowed free access to the control diet or a diet containing 50 g LPC/kg for 1 or 3 d. Eight rats in each group were killed by decapitation without prior food deprivation, and the stomach and the small intestine were excised. Mucosal sampling for RNA isolation was performed as described for *Expt. 2*. For the remaining rats (6 rats per group), diets were withdrawn overnight and rats were then killed by decapitation. Luminal mucin sampling and collection of intestinal tissue were performed as described for *Expt. 1*.

Expt. 4 Mucin-secreting HT29-MTX cells established from the cultured human colonic adenocarcinoma parental HT-29 cell line were kindly provided by Dr Thécla Lesuffleur (INSERM UMR S 938, Paris, France) (25). Cells were maintained in Dulbecco's modified Eagle's minimal essential medium (**DMEM**) (Sigma, St Louis, MO) supplemented with 10% inactivated fetal bovine serum (Gibco, Grand Island, NY) and 1% antibiotic-antimycotic solution (Antibiotic-Antimycotic Solution; 1.0×10^7 units of penicillin, 10,000 mg of streptomycin, and 25 mg of amphotericin

B /L, Gibco) at 37°C in a 10% CO_2 /90% air atmosphere. In order to assess the mucin-secretory effects of dietary fibers, the cells were seeded at 1.0×10^6 cells in 6-well plates (NUNC, Roskilde, Denmark) and pre-cultured for 7 d. Cells were washed with PBS three times and cultured with 1 mL of DMEM with 1% media supplement (Insulin-Transferrin-Sodium Selenite Supplement; 10 mg of human insulin, 5.5 mg of human transferrin, and 5.5 mg of sodium selenite /L, SIGMA) in the presence of 100 mg /L of soluble dietary fiber (LPC, HPC, GGH, GGL, KMH, KML, SAL, or LAG) for 24 h. Lipopolysaccharide (1.0 mg/L, **LPS**) was used as a positive control (30). The culture media were gathered, and the cells were washed with 1 mL of PBS. The combined solution (culture media + washings) was centrifuged at 10,000×*g* for 10 min and the supernatant was subjected to MUC5AC ELISA. Dose-dependent effects of LPC and HPC (0, 25, 50, 100, 200 mg /L) on mucin secretion were examined using the same procedures described above.

Expt. 5 After acclimatization for 5 d, 48 Wistar rats, weighing 132-162 g (6 wk old), were divided into 4 groups of 12 rats and allowed free access to the control diet or a diet containing 50 g of LPC/kg with or without NM (1 g/L) in drinking water for 10 d. There were thus four groups: control, LPC, control+NM, and LPC+NM (2 x 2 factorial design). Half of the rats in each group were killed by decapitation without prior food deprivation, and the small intestine and the cecum were excised. Mucosal sampling from the small intestine for RNA isolation was as described for *Expt. 2*. The cecal contents were weighed and used for measurements of organic acids. For the

remaining rats, diets were withdrawn overnight and the rats were killed by decapitation. Luminal mucin sampling procedures and the intestinal tissue collection were conducted in the same manner as in *Expt. 1*.

Luminal mucin analysis. The luminal mucin fraction was isolated using the method of Lien et al. (31) with some modification, as described previously (16), and was dissolved in 5.0 mL of distilled water for analyses. After appropriate dilution of the mucin fraction, luminal mucin-ELISA was performed as described previously (32).

Goblet cell staining. Six 5-μm-thick cross-sections were prepared per rat from paraffin-embedded samples and first stained with periodic acid Schiff, then counter-stained with hematoxylin (18).

RNA isolation and quantitative RT-PCR. Total RNA isolation and quantitative RT-PCR were performed as previously described (19). The primer pairs and protocols for PCR of *Muc2* (33), *Muc3* (33), *Muc5ac* (34) and 18S rRNA (35) have been previously reported. 18S rRNA was used as an endogenous reference gene. To confirm amplification specificity, the PCR products from each primer pair were subjected to a melting curve analysis. Gene expression was quantified using the comparative CT method (36), and the data were expressed relative to the control group.

MUC5AC analysis. The supernatant was appropriately diluted with 50 mmol/L sodium carbonate buffer (pH 9.6) and plated overnight at 4°C on 96-well plates. Unbound sites were blocked with 1% skim milk in PBS. The plates were washed with PBS containing 0.05% Tween 20 and subsequently incubated with anti-human MUC5AC antibody (Clone

1-13M1 mAb, Abcam, Cambridge, UK) for 2 h at 37°C. Following a second washing procedure, peroxidase-conjugated anti-mouse antibody (Sigma) was applied for 1 h at 37°C. Plates were washed and developed using 3, 3', 5, 5'-tetramethylbenzidine. The reaction was terminated with 1 mol/L H_2SO_4 and the absorbance at 450 nm was measured. MUC5AC mucin was purified from the culture media of HT-29MTX cells by Sepharose 4B gel chromatography (GE Health Care Japan, Tokyo, Japan), and used as a control.

Organic acid quantification. Cecal organic acids (formate, acetate, propionate, *iso*-butyrate, *n*-butyrate, *iso*-valerate, *n*-valerate, succinate and lactate) were measured by the internal standard method using an HPLC equipped with Shim-pack SCR-102H column (Shimadzu, Kyoto, Japan) and an electroconductivity detector (CDD-6A, Shimadzu) (29).

Statistical analyses. Data were analyzed by one-way (*Expt. 1, 2* and *4*) or two-way ANOVA (*Expt. 5*), or two-way Repeated Measures ANOVA (*Expt. 3*). Differences between the groups were analyzed by the Tukey-Kramer test (*Expt. 1, 2* and *4*). In *Expt. 4* (Fig. 1-B), a linear regression analysis was conducted to examine whether there was a dose-response between pectin and MUC5AC release. The results were expressed as means ± SEM and a 5% level of probability was considered significant for all analyses. The statistical calculations were carried out using JMP8 software (SAS Institute, Cary, NC).

Results

LPC ingestion stimulates small intestinal mucin secretion irrespective of goblet cell proliferation (*Expt. 1*).

Food intakes in the KMH and KML groups were significantly lower than in the control, LPC and SAL groups (**Table 1**). In addition, the KMH group gained significantly less weight than the other groups. The amount of mucin in the small intestine was greater in the LPC and KMH groups than the control and other fiber-diet groups, although there were no significant differences compared to the GAL group. The KMH group had a greater number of ileal goblet cells compared to all other groups.

Ingestion of LPC leads to up-regulation of jejunal *Muc2* expression (*Expt. 2*).

Food intake in the KMH group was significantly lower than in the control and LPC groups, but body weight gain did not differ among the groups (**Table 2**). The amount of mucin in the small intestine was significantly greater in the LPC and KMH groups compared to the control and HPC groups. In the jejunum, the KMH group had significantly greater villus length compared to the LPC group and more goblet cells than the other groups. Similarly, ileal villus length was greatest in the KMH group and lowest in the LPC group, with intermediate values in the control and HPC groups, and all differences were significant. The number of ileum goblet cells in the KMH group was significantly greater than in the other groups. *Muc5ac* expression in the stomach was comparable among the groups. Jejunal *Muc2* expression was significantly higher in the LPC group compared to all other groups, although *Muc2* expression in the ileum did

not differ among the groups. There were no differences in jejunal and ileal *Muc3 gene* expression among the groups.

Ingestion of LPC up-regulates jejunal *Muc2* expression within a day (*Expt. 3*).

Food intake in the LPC group for 1 and 3 d was lower than in the control group, while body weight gain did not differ between the groups at both time points (**Table 3**). Luminal mucin was greater in the LPC group than in the control group at both time points. *Muc5ac* expression in the stomach was similar in the two groups at both time points. The LPC group had greater *Muc2* and *Muc3* expression in the jejunum than the control group at both time points. In the ileum, *Muc2* expression in the LPC group was greater than the control group, while *Muc3* expression was similar in the two groups at both time points. As for luminal mucin and gene expression, only the diet effect was observed.

LPC and HPC stimulate mucin secretion from HT-29MTX cells (*Expt. 4*).

Exposure of HT-29 MTX cells to various soluble fibers showed that LPC and HPC significantly increased mucin secretion by 72% (LPC) and 59% (HPC), respectively, compared with the control, but other soluble fibers did not (**Fig. 1 A**). The mucin-secretory effects of LPC and HPC were comparable to that of LPS that was used as a positive control. LPC increased mucin secretion in a dose-dependent manner when the range was limited to 0-100 mg/L ($r = 0.96$, $P < 0.001$), and the effect of LPC was

significant at 100 mg/L compared with the control. However, the effect of LPC disappeared at high concentrations (200 mg/L). HPC did not show a dose-dependent effect, but mucin secretion significantly increased at 50 mg/L ($P = 0.02$) and tended to increase at 100 mg/L ($P = 0.051$) (**Fig. 1 B**).

Neomycin treatment does not affect mucin-secretory effects of LPC (*Expt. 5*).

Food intake and body weight gain did not differ among the groups (**Table 4**). Small intestinal mucin contents were significantly affected by the LPC treatment, and NM treatment did not affect the results. In the jejunum, *Muc2* expression was significantly affected by the LPC treatment, while NM treatment did not affect the results. *Muc3* expression did not differ among the groups. In the ileum, neither *Muc2* nor *Muc3* expression differed among the groups. Cecal concentrations of total organic acids and SCFA were significantly affected by the treatment with LPC. NM treatment also had a significant effect on the cecal fermentation, but there were no significant interactions between the treatments.

Discussion

In contrast to diets containing KMH, a high-viscosity fiber that was shown to induce goblet cell proliferation and luminal mucin secretion, ingestion of low-viscosity fibers (GGL, KML, SAL, GAL and LAG) did not affect goblet cells or luminal mucins in the present study. However, ingestion of LPC diet resulted in increased mucin secretion irrespective of goblet cell

number. These findings are in accordance with previously published results (18) and strongly suggest that the mechanism for the stimulatory effect of LPC on mucin secretion differs from that of high-viscosity soluble fibers. Although Kishioka et al. (21) reported that an alginate, exo-polysaccharide secreted by *Pseudomonas aeruginosa*, stimulated mucin secretion from the isolated ferret trachea, we observed no increase in luminal mucins in rats fed the SAL diet in the present study. The reason for the difference in response to alginate is unknown.

In the present study, we showed that ingestion of the LPC, but not KMH or HPC diet, for 10 d increased *Muc*2 expression in the jejunum. Furthermore, ingestion of the LPC diet for only 1 d was sufficient to induce significant increases in *Muc2 and Muc3* expression in the jejunum. The intestinal epithelium is renewed with an average turnover rate of 3-5 d (2). The previous study showed that the effect on luminal mucin secretion by bulky fiber ingestion was connected with epithelial cell turnover (16, 17), i.e., goblet cell proliferation, and appeared or disappeared within 5 d after starting or ceasing the fiber ingestion (17). An early response of jejunal *Muc*2 expression to the LPC diet may therefore suggest that the LPC molecule has a direct effect on the epithelium and enhances mucin discharge from individual goblet cells.

Interestingly, increased *Muc* gene expression following ingestion of the LPC diet was observed predominantly in the jejunum (*Expt.* 2 and *3*). We suggest that this site-specific effect of LPC on *Muc2* expression is due

primarily to the difference in the physical state of LPC lying in the jejunal and ileal contents. Free carboxyl groups of pectin form gels through the action of dicationic ion, which cross-links the galacturonic acid chains (20). Dintzis et al. (37) showed in pigs that the luminal concentration of dicationic ion was elevated along the length of the small intestine due to a gradual absorption of luminal water. Accordingly, promotion of gel-formation in the ileum would reduce the amount of LPC molecules in the free form that are available to interact with the epithelial tissue.

To further examine a direct interaction of LPC with the epithelium, we conducted a cell culture study using the HT29-MTX cell line, which is a well-characterized mucin-secreting cell subpopulation. Among the various soluble fibers tested, only LPC and HPC induced a significant increase in mucin release from the cells. Treatment with LPC in particular resulted in a dose-dependent stimulation of mucin release up to a final concentration of 100 mg/L in the culture medium. With respect to LPC, the results from the cell culture study support the findings from the rat experiments (*Expt. 1*, *2* and *5*) where a specific effect of LPC ingestion on mucin secretion was observed. In contrast, the effect of HPC on mucin secretion differed between *in vitro* and *in vivo* conditions. At present, the reason for this is unclear, but may be partially explained by a sol-gel transition property of this molecule. HPC forms a gel by cross-linking of hydrogen bridges and by hydrophobic forces between methoxyl groups. Both bonds are promoted in a lower pH environment (20). The physicochemical characteristics of HPC suggest the possibility that HPC may form a gel in the stomach before

reaching the intestine, thereby losing its effect on the intestinal epithelium. This is unlikely to occur *in vitro* because HPC in the medium is completely dispersed.

Finally, the possible contribution of SCFA to mucin secretion following LPC ingestion was examined. Despite reduced SCFA production, the stimulatory effects of LPC on jejunal *Muc2* expression and luminal mucin secretion were not affected, clearly indicating that SCFA are not involved in the mucin-secretory effect of LPC in the small intestine.

Although the chemical structure required for the mucin-secretory effect of LPC remains unclear, the present findings implicate that a uronic acid polymer *per se* is not a necessary prerequisite for the *in vivo* effect of LPC on mucin secretion. Further study is needed to clarify this. Nevertheless, the results from the present study clearly indicate that the mechanism of mucin stimulation by LPC differs from that of high-viscosity fibers and that the LPC molecule has the ability to interact directly with epithelial tissue in the intestine and enhance mucin discharge from individual goblet cells accompanied by *Muc2* up-regulation. Thus, we propose that LPC acts as a mucin secretagogue in the rat small intestine and that this effect is mediated by “regulated secretion”.

Acknowledgement

T.M. designed the research and wrote the manuscript; S.H., H.B., and K.S. conducted the research, and H.K., and S.A. analyzed the data. All authors read and approved the final manuscript. We express our thanks to Dr Thécla Lesuffleur (INSERM UMR S 938, Paris, France) for the kind gift of the HT-29 MTX cell-line

Literature Cited

1. Deplancke B, Gaskins HR. Microbial modulation of innate defense: goblet cells and the intestinal mucus layer. Am. J. Clin. Nutr. 2001; 73: 1131S-1141S.
2. Specian RD, Oliver MG. Functional biology of intestinal goblet cells. Am. J. Physiol. 1991; 260: C183-193.
3. Van M, De KBA, De BAC, Velcich A, Meijerink JP, Van GJB, Buller HA, Dekker J, Van SI, Renes IB, Einerhand AW. Muc2-deficient mice spontaneously develop colitis, indicating that MUC2 is critical for colonic protection. Gastroenterology. 2006; 131: 117-129.
4. Pullan RD, Thomas GA, Rhodes M, Newcombe RG, Williams GT, Allen A, Rhodes J. Thickness of adherent mucus gel on colonic mucosa in humans and its relevance to colitis. Gut. 1994; 35: 353-359.
5. Faure M, Moennoz D, Montigon F, Mettraux C, Breuille D, Ballevre O. Dietary threonine restriction specifically reduces intestinal mucin synthesis in rats. J. Nutr. 2005; 135: 486-491.
6. Claustre J, Toumi F, Trompette A, Jourdan G, Guignard H, Chayvialle JA, Plaisancie P. Effects of peptides derived from dietary proteins on mucus secretion in rat jejunum. Am. J. Physiol. Gastrointest. Liver. Physiol. 2002; 283: G521-528.
7. Cassidy MM, Lightfoot FG, Grau LE, Story JA, Kritchevsky D, Vahouny GV. Effect of chronic intake of dietary fibers on the ultrastructural topography of rat jejunum and colon: a scanning electron microscopy study. Am. J. Clin. Nutr. 1981; 34: 218-228.

8. Vahouny GV, Le T, Ifrim I, Satchithanandam S, Cassidy MM. Stimulation of intestinal cytokinetics and mucin turnover in rats fed wheat bran or cellulose. Am. J. Clin. Nutr. 1985; 41: 895-900.
9. Toden S, Bird AR, Topping DL, Conlon MA. Resistant starch attenuates colonic DNA damage induced by higher dietary protein in rats. Nutr. Cancer. 2005; 51: 45-51.
10. Toden S, Bird AR, Topping DL, Conlon MA. Dose-dependent reduction of dietary protein-induced colonocyte DNA damage by resistant starch in rats correlates more highly with caecal butyrate than with other short chain fatty acids. Cancer. Biol. Ther. 2007; 6: 253-258.
11. Shimotoyodome A, Meguro S, Hase T, Tokimitsu I, Sakata T. Short chain fatty acids but not lactate or succinate stimulate mucus release in the rat colon. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 2000; 125: 525-531.
12. Barcelo A, Claustre J, Moro F, Chayvialle JA, Cuber JC, Plaisancie P. Mucin secretion is modulated by luminal factors in the isolated vascularly perfused rat colon. Gut. 2000; 46: 218-224.
13. Burger-van PN, Vincent A, Puiman PJ, van M, Bouma J, Boehm G, van GJB, van SI, Renes IB. The regulation of intestinal mucin MUC2 expression by short-chain fatty acids: implications for epithelial protection. Biochem. J. 2009; 420: 211-219.
14. Zoghbi S, Trompette A, Claustre J, El HM, Garzon J, Jourdan G, Scoazec JY, Plaisancie P. beta-Casomorphin-7 regulates the secretion and expression of gastrointestinal mucins through a mu-opioid pathway. Am. J. Physiol. Gastrointest. Liver. Physiol. 2006; 290: G1105-1113.

15. Gaudier E, Rival M, Buisine MP, Robineau I, Hoebler C. Butyrate enemas upregulate Muc genes expression but decrease adherent mucus thickness in mice colon. Physiol. Res. 2009; 58: 111-119.
16. Tanabe H, Sugiyama K, Matsuda T, Kiriyama S, Morita T. Small intestinal mucins are secreted in proportion to the settling volume in water of dietary indigestible components in rats. J. Nutr. 2005; 135: 2431-2437.
17. Morita T, Tanabe H, Ito H, Yuto S, Matsubara T, Matsuda T, Sugiyama K, Kiriyama S. Increased luminal mucin does not disturb glucose or ovalbumin absorption in rats fed insoluble dietary fiber. J. Nutr. 2006; 136: 2486-2491.
18. Ito H, Satsukawa M, Arai E, Sugiyama K, Sonoyama K, Kiriyama S, Morita T. Soluble fiber viscosity affects both goblet cell number and small intestine mucin secretion in rats. J. Nutr. 2009; 139: 1640-1647.
19. Hino S, Takemura N, Sonoyama K, Morita A, Kawagishi H, Aoe S, Morita T. Small intestinal goblet cell proliferation induced by ingestion of soluble and insoluble dietary fiber is characterized by an increase in sialylated mucin in rats. J. Nutr. 2012; 142: 1429-1436.
20. Thakur BR, Singh RK, Handa AK. Chemistry and uses of pectin - a review. Crit. Rev. Food. Sci. Nutr. 1997; 37: 47-73.
21. Kishioka C, Okamoto K, Hassett DJ, de MD, Rubin BK. Pseudomonas aeruginosa alginate is a potent secretagogue in the isolated ferret trachea. Pediatr. Pulmonol. 1999; 27: 174-179.

22. Dongowski G, Lorenz A, Proll J. The degree of methylation influences the degradation of pectin in the intestinal tract of rats and in vitro. J. Nutr. 2002; 132: 1935-1944.
23. Fukunaga T, Sasaki M, Araki Y, Okamoto T, Yasuoka T, Tsujikawa T, Fujiyama Y, Bamba T. Effects of the soluble fibre pectin on intestinal cell proliferation, fecal short chain fatty acid production and microbial population. Digestion. 2003; 67: 42-49.
24. Satchithanandam S, Vargofcak-Apker M, Calvert RJ, Leeds AR, Cassidy MM. Alteration of gastrointestinal mucin by fiber feeding in rats. J. Nutr. 1990; 120: 1179-1184.
25. Lesuffleur T, Barbat A, Dussaulx E, Zweibaum A. Growth adaptation to methotrexate of HT-29 human colon carcinoma cells is associated with their ability to differentiate into columnar absorptive and mucus-secreting cells. Cancer. Res. 1990; 50: 6334-6343.
26. Prosky L, Asp NG, Schweizer TF, DeVries JW, Furda I. Determination of insoluble, soluble, and total dietary fiber in foods and food products: interlaboratory study. J. Assoc. Off. Anal. Chem. 1988; 71: 1017-1023.
27. Dikeman CL, Murphy MR, Fahey GCJ. Dietary fibers affect viscosity of solutions and simulated human gastric and small intestinal digesta. J. Nutr. 2006; 136: 913-919.
28. Olmos JC, Hansen MEZ. Enzymatic depolymerization of sugar beet pulp: production and characterization of pectin and pectic-oligosaccharides as a potential source for functional carbohydrates. Chem. Eng. J. 2012; 192: 29-36.

29. Morita T, Kasaoka S, Ohhashi A, Ikai M, Numasaki Y, Kiriyama S. Resistant proteins alter cecal short-chain fatty acid profiles in rats fed high amylose cornstarch. J. Nutr. 1998; 128: 1156-1164.
30. Smirnova MG, Guo L, Birchall JP, Pearson JP. LPS up-regulates mucin and cytokine mRNA expression and stimulates mucin and cytokine secretion in goblet cells. Cell. Immunol. 2003; 221: 42-49.
31. Lien KA, McBurney MI, Beyde BI, Thomson AB, Sauer WC. Ileal recovery of nutrients and mucin in humans fed total enteral formulas supplemented with soy fiber. Am. J. Clin. Nutr. 1996; 63: 584-595.
32. Tanabe H, Ito H, Sugiyama K, Kiriyama S, Morita T. Estimation of luminal mucin content in rats by measurement of O-linked oligosaccharide chains and direct ELISA. Biosci. Biotechnol. Biochem. 2007; 71: 575-578.
33. Tsuboi Y, Kim Y, Paparella MM, Chen N, Schachern PA, Lin J. Pattern changes of mucin gene expression with pneumococcal otitis media. Int. J. Pediatr. Otorhinolaryngol. 2001; 61: 23-30.
34. Ishibashi Y, Kobayashi F, Idesawa A, Taniguchi A, Matsuzawa S. Effects of carbocisteine on altered activities of glycosidase and glycosyltransferase and expression of Muc5ac in SO2-exposed rats. Eur. J. Pharmacol. 2004; 487: 7-15.
35. Zhu LJ, Altmann SW. mRNA and 18S-RNA coapplication-reverse transcription for quantitative gene expression analysis. Anal. Biochem. 2005; 345: 102-109.
36. Heid CA, Stevens J, Livak KJ, Williams PM. Real time quantitative PCR. Genome. Res. 1996; 6: 986-994.

37. Dintzis FR, Laszlo JA, Nelsen TC, Baker FL, Calvert CC. Free and total ion concentrations in pig digesta. J. Anim. Sci. 1995; 73: 1138-1146.

Figure legend

Fig. 1 Mucin release from HT-29MTX cells treated with various soluble fibers at 100 mg/L (A) or various concentrations of LPC and HPC (B)(*Expt.* 4).

Data are mean ± SE, n=3 (A) or 4 (B). Means for a variable without a common letter differ, $P < 0.05$. GGH, guar gum of high molecular weight; GGL, guar gum of low molecular weight; HPC, high-methoxyl pectin; KMH, konjac mannan of high molecular weight; KML, konjac mannan of low molecular weight; LAG, larch arabinogalactan; LPC, low-methoxyl pectin; LPS, lipopolysaccharide; SAL, sodium alginate of low molecular weight.

Table 1 Food intake, body weight gain, small intestinal mucin content, and goblet cell number in the ileum in Wistar rats fed the control diet, or diets containing 50 g /kg of various soluble fibers for 10 d (*Expt.* 1) [1]

	Control	LPC	GGL	KML	SAL	GAL	LAG	KMH
Food intake, *g/10 d*	149±4 [a]	147±3 [a]	146±4 [ab]	137±4 [b]	150±3 [a]	146±5 [ab]	142±3 [ab]	119±3 [c]
Body weight gain, *g/10 d*	50±2 [a]	48±2 [a]	48±2 [a]	46±3 [a]	47±2 [a]	51±2 [a]	49±2 [a]	38±2 [b]
Small intestinal mucin, *mg*	1.0±0.1 [b]	1.5±0.2 [a]	0.7±0.1 [b]	1.0±0.2 [b]	0.9±0.1 [b]	1.0±0.2 [ab]	0.8±0.1 [b]	1.4±0.2 [a]
Ileal goblet cells, *n/villus, left side*	10.7±0.7 [b]	10.6±0.7 [b]	11.1±0.6 [b]	10.5±0.4 [b]	10.8±0.3 [b]	11.9±0.4 [b]	10.0±0.3 [b]	14.4±0.7 [a]

[1] Values are mean ± SE, $n = 8$. Means in a row without a common letter differ, $P < 0.05$. GAL, gum arabic of low molecular weight; GGL, guar gum of low molecular weight; KMH, konjac mannan of high molecular weight; KML, konjac mannan of low molecular weight; LAG, larch arabinogalactan; LPC, low-methoxyl pectin; SAL, sodium alginate of low molecular weight.

Table 2 Food intake, body weight gain, small intestinal mucin content, villus length and goblet cell number in the jejunum and ileum, and *Muc* gene expression in the stomach, jejunum and ileum in Sprague-Dawley rats fed the control diet, or a diet containing 50 g /kg of HPC, LPC or KMH for 10 d (*Expt.* 2) [1]

	Control	HPC	LPC	KMH
Food intake, *g/10 d*	161 ± 3 [a]	149 ± 3 [bc]	158 ± 3 [ab]	141 ± 2 [c]
Body weight gain, *g/10 d*	82 ± 2	75 ± 2	80 ± 2	75 ± 2
Small intestinal mucin, *mg*	1.2±0.2 [b]	1.3±0.2 [b]	2.3±0.3 [a]	2.5±0.5 [a]
Jejunum				
Villus length, *μm*	447±16 [ab]	423±8 [ab]	399±21 [b]	456±15 [a]
Goblet cells, *n/villus, left side*	11.1±0.3 [b]	10.8±0.4 [b]	11.4±0.2 [b]	11.7±0.2 [a]
Ileum				
Villus length, *μm*	390±4 [b]	385±9 [b]	335±7 [c]	414±14 [a]
Goblet cells, *n/villus, left side*	10.3 ± 0.1 [b]	10.3 ± 0.1 [b]	10.4 ± 0.1 [b]	13.9 ± 0.2 [a]
Muc gene expression				
Stomach				
Muc5ac	1.0 ± 0.1	1.1 ± 0.2	1.3 ± 0.2	1.0 ± 0.3
Jejunum				
Muc2	1.0 ± 0.1 [b]	1.0 ± 0.1 [b]	1.6 ± 0.2 [a]	1.1 ± 0.1 [b]
Muc3	1.0 ± 0.1	1.1 ± 0.2	1.5 ± 0.3	1.6 ± 0.2
Ileum				
Muc2	1.0 ± 0.1	0.7 ± 0.1	0.9 ± 0.1	1.2 ± 0.1
Muc3	1.0 ± 0.1	0.7 ± 0.1	0.7 ± 0.2	0.9 ± 0.1

[1] Values are mean ± SE, $n = 8$ or 16 (food intake, body weight gain). Means in a row without a common letter differ, $P < 0.05$. HPC, high-methoxyl pectin; KMH, konjac mannan of high molecular weight; LPC, low-methoxyl pectin.

Table 3 Food intake, body weight gain, small intestinal mucin content, and *Muc* gene expression in the stomach, jejunum and ileum in Sprague-Dawley rats fed the control diet or a diet containing 50 g /kg of LPC for 1 or 3d (*Expt.* 3) [1]

	1d		3d		Two-way Repeated Measures ANOVA		
	Control	LPC	Control	LPC	Diet	Time	Interaction
Food intake, *g*	19± 1	16 ± 1	56 ± 2	51 ± 1	<0.01	<0.01	NS [2]
Body weight gain, *g*	9 ± 1	9 ± 1	25 ± 1	25 ± 1	NS	<0.01	NS
Small intestinal mucin, *mg*	1.0 ± 0.1	1.2 ± 0.1	1.0 ±0.1	1.2 ± 0.1	<0.01	NS	NS
Muc gene expression							
Stomach							
Muc5AC	1.0 ± 0.1	1.1 ± 0.2	1.0 ± 0.1	1.0 ± 0.1	NS	NS	NS
Jejunum							
Muc2	1.0 ± 0.1	1.5 ± 0.1	1.0 ± 0.1	1.4 ± 0.1	<0.01	NS	NS
Muc3	1.0 ± 0.2	1.6 ± 0.2	1.0 ± 0.2	1.6 ± 0.2	<0.01	NS	NS
Ileum							
Muc2	1.0 ± 0.2	1.2 ± 0.1	1.0 ± 0.1	1.2 ± 0.2	<0.01	NS	NS
Muc3	1.0 ± 0.2	0.9 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	NS	NS	NS

[1] Values are mean ± SE, n =6 (small intestinal mucin), 8 (*Muc* gene expression) or 14 (food intake, body weight gain) and analyzed using two-way Repeated Measures ANOVA. LPC, low-methoxyl pectin.

[2] Not significant, $P > 0.05$.

Table 4 Food intake, body weight gain, small intestinal mucin content, *Muc* gene expression in the jejunum and ileum, and total organic acids and SCFA concentrations in the cecum in Wistar rats fed the control diet or a diet containing 50 g /kg of LPC with or without NM (1 g /L) in drinking water for 10 d (*Expt.* 5) [1]

					Two-way ANOVA		
	Control	LPC	Control + NM	LPC + NM	LPC	NM	Interaction
Food intake, *g/10 d*	140 ± 6	142 ± 5	139 ± 8	128 ± 3	NS [2]	NS	NS
Body weight gain, *g/10 d*	51 ± 2	50 ± 2	53 ± 3	44 ± 4	NS	NS	NS
Small intestinal mucin, *mg*	1.1 ± 0.1	1.4 ± 0.1	1.0 ± 0.1	1.5 ± 0.2	< 0.05	NS	NS
Muc gene expression							
Jejunum							
Muc2	1.0 ± 0.1	1.5 ± 0.2	1.0 ± 0.1	1.6 ± 0.1	< 0.01	NS	NS
Muc3	1.0 ± 0.1	1.4 ± 0.3	1.4 ± 0.2	1.9 ± 0.4	NS	NS	NS
Ileum							
Muc2	1.0 ± 0.1	1.1 ± 0.1	1.1 ± 0.2	1.2 ± 0.1	NS	NS	NS
Muc3	1.0 ± 0.1	1.1 ± 0.3	1.6 ± 0.4	1.1 ± 0.5	NS	NS	NS
Cecum							
Total organic acids, *μmol/g contents*	87 ± 5	126 ± 6	34 ± 3	72 ± 8	< 0.01	< 0.01	NS
SCFA *μmol/g contents* [3]	84 ± 5	122 ± 7	16 ± 2	42 ± 6	< 0.01	< 0.01	NS

[1] Values are mean ± SE, $n = 6$ or 12 (food intake, body weight gain) and analyzed using two-way ANOVA. LPC, low-methoxyl pectin; NM, neomycin; SCFA, short-chain fatty acids.

[2] Not significant, $P > 0.05$.

[3] Sum of acetate, propionate, and *n*-butyrate.

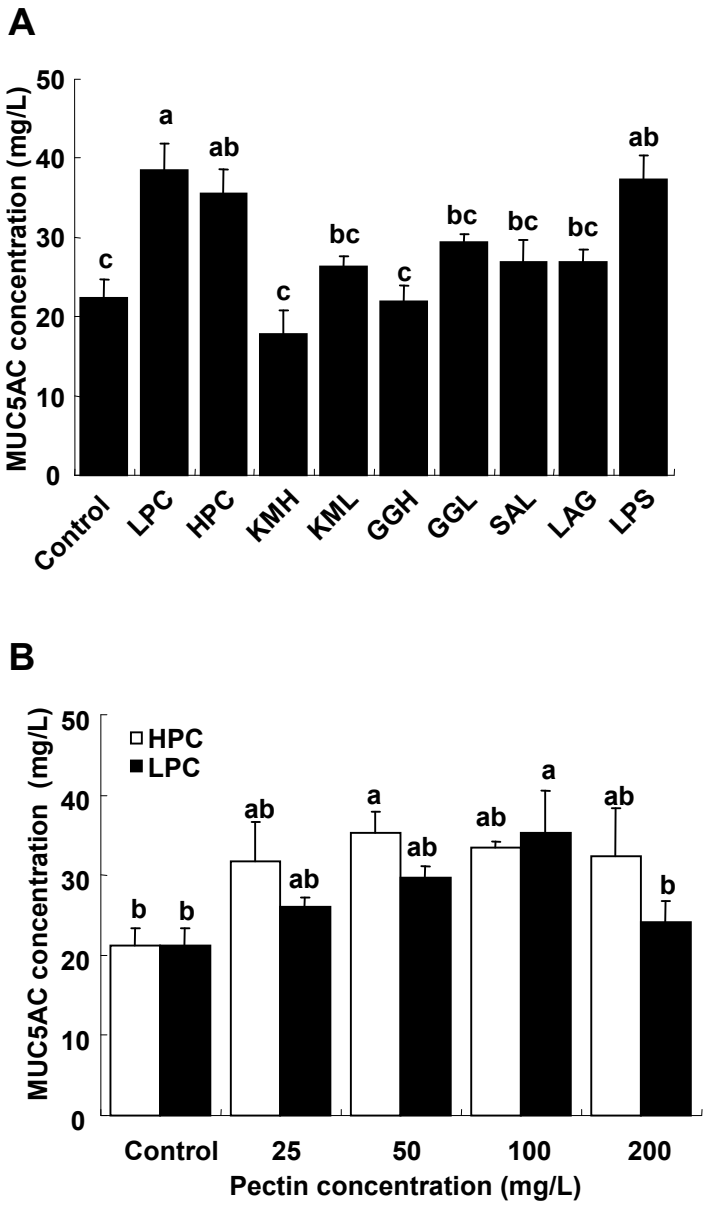
A
MUC5AC concentration (mg/L)
0
10
20
30
40
50
c
a
ab
c
bc
c
bc
bc
bc
ab
Control
LPC
HPC
KMH
KML
GGH
GGL
SAL
LAG
LPS
B
MUC5AC concentration (mg/L)
HPC
LPC
b
b
ab
ab
a
ab
ab
a
ab
b
Control
25
50
100
200
Pectin concentration (mg/L)

Figure 1