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Increasing Effect of a Chitosan and Ascorbic Acid Mixture on Fecal Dietary Fat Excretion

Osamu KANAUCHI, Kejji DEUCHI, Youji IMASATO, and Eiichi KOBAYASHI

Applied Research Center, Research and Development Dept., Kirin Brewery Co Ltd., 3 Miyahora-cho, Tokasaki-shi, Gunma 370-12 Japan

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Rats were fed for2 weeks on five different high-fat diets containing cellulose (control) or chitosan with and without organic acids (i.e., ascorbic, lactic and citric acids) as the dietary fiber. The **apparent fat digestibility in the chitosan-receiving groups was significantly lower than in the control group. The addition of ascorbic acid to chitosan caused a larger increase in the fecal fat excretion than otherwise without considerably affecting the apparent protein digestibility.**

The effect is thought to have occurred because chitosan was so well dissolved and mixed with fat by the action of ascorbic acid that it encapsulated fine fat droplets on its gel after contacting the pancreatic juice in the small intestine. The intake of mixture of chitosan and ascorbic acid is suitable for reducing excess fat.

Chitosan is an N-deacetylated product of chitin, and its basic structure is (1-4) linked 2-amino-2-deoxy-B-o-glucan. Chitosan has been shown to have potent hypocholesterolemic effect and physiological functions. In particular, the hypocholesterolemic effect of chitosan has been extensively studied.

The most acceptable explanation for the effect of chitosan interacts with acidic and neutral steroids in the intestinal lumen and increases their fecal excretion.

We have previously reported that chitosan caused a marked increase in the fecal excretion of dietary fat given to rats, although chitin, which in a sense is "acetylated chitosan", did not have such an effect.*

Chitosan is easy to dissolve in dilute HCI (e.g. gastric acid) and in certain organic acids, in contrast to chitin The difference in fecal fat excretion between chitosan and chitin may bee accounted for by their different solubility in the gastric phase (acidic media).

In order to elucidate this possibility, chitosan supplemented with organic acids was fed to rats, and the extent of the apparent digestibility of dietary fat was examined.

Materials and Methods

Materials. Chitosan was purchased from Katakura-Chikkarin Co. (the viscosity was about 200-250 cps and the deacetylation value was more the 90%). Ascorbic acid, citric acid, and lactic acid were purchased from Wako Pure Chemicals Co. The dietary components except for corn oil (Ajimamoto Co.), were purchased from Oriental Yeast co.

Animal and treatment. Male Sprague-Dawley rats weighing 130-15Og were purchased from Charles River Japan. They were individually housed in metabolic cages in a room kept at 22+/- 1-C with a 12-hour light and dark cycle (lighting from 8:00 a.m. to 8:0O p.m.).

The rats were fed ad libitum with a commercial diet for 5 days, and were then assigned to several groups (n=8-10). One group received cellulose as the dietary fiber (control), and the others received chitosan instead of cellulose. The latter were divided into 4 subgroups, each being respectively fed with chitosan without organic acids (CHI group), and supplemented with ascorbic acid (CHI+AsA group), citric acid (CHI+ CIT group). or lactic acid (CHI+LAC group) We chose sucrose as the sole carbohydrate source because l) many researchers who have studied dietary fibers', and especially chitosan used sucrose according to the AlN76 formula. and 2) we were worried that undigested starch fragments would also serve as dietary fiber and complete with chitosan for fat trapping The compositions of the diets used in this experiment are shown in Table I

During the experimental period, each rat was allowed free access to food and tap water On days 0, 2, 4 and 10, 20 ul of blood was drawn from the tail vein by using a heparinized capillary tube The feces were collected on days 11, 12, and 13, and after the feces has been collected on day 13, all food was withdrawn at 11:00 The rats were then fasted for about 24 hour, and on day 14, were anesthetized with urethane to excise the liver and epididymal fat pads

Methods The feces collected from each animal were lyophilized, pulverized and weighted. The fecal samples thus obtained were analyzed for their fat and protein contents, and for their fatty acid composition according to a modification of the Saxton Gravity method"' described in the previous study. The apparent fat digestibility was calculated as follows: [(fat intake -- fecal fat)/fat intake] x 100 (%)

The fecal nitrogen content was measured by the Kjeldahl method, and after analyzing the apparent protein digestibility, was calculated by using the following equation [(nitrogen intake -- fecal nitrogen) / (nitrogen intake - nitrogen in chitosan)] x 100 (%)

The composition of fatty acids was determined by gas-liquid chromatography (GLC) under the same conditions as those described previously In brief, the fecal samples were extracted with chloroform-methanol (2:1, v/v) according to the method of Folch et al, and after removing the solvent under a nitrogen stream, the lipids were transmethylated with methanol-BF„ and the fatty acid methyl esters were. then subjected to GLC Undecanoic acid was used as an Internal standard

Table I. Composition of the Diets (%)

Constituent	Control	Chitosan (CHI) alone	+ASA	/LAC	+CIT
Casein	20	20	20	20	20
Corn Oil	20	20	20	20	20
Mineral Mix ¶	3.5	3.5	3.5	3.5	3.5
Vitamin mix **	1	1	1	1	1
Cholinme chl.	0.2	0.2	0.2	0.2	0.2
Chitosan (CHI)***		5	5	5	5
Cellulose	5				
Ascorbic Acid			1.5		
Lactic acid				1.5	
Citric Acid					1.5
Sucrose	50.3	50.3	48.8	48.8	48.8

* The mineral mixture was prepared according to the AIN-76 specifications."

• * The vitamin mixture was prepared according to the AIN-76 specification.'"

• ** Viscosity was about 200-250 cps (measured by using a 0.5% solution of dried materials in 0.5% acetic acid at 25'C with a rotary viscometer (Tokyo Keiki Seisakusyo, Type B). The degree of deacetylation of chitosan was more than 90%.

1 *K. Deuchi, O. Karauchi, Y. Imasaio, and E. Kobayashi *Biosci, Biotech, Biochem.*, 58 (9), 1617-1620, T994

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Blood (20μl) collcted on days 0 ,2 ,4, and 10 was separated into the plasma by cenrifugation at 8000 rpm for 7 min. and its triacylglycerol (TG) concentration was determined by using a commercial assay kit (TG-EN KAINOS, KAINOS Co.)

The viscosity of chitosan was analyzed by using aa type B rotary viscosity meter (Tokyo Keiki Co.) At 37°C, which is similar to the rat body temperature. Chitosan (1.5g) was dissolved in 300 ml of 0.lM HCl ascorbic acid, citric acid or lactic acid and then incubated at 37°C for 3 hours. The viscosity of the chitosan-acid solution was then measured. To examine how the viscosity of chitosan in HCl would be affected by the addition of different kinds of organic acid, 1% of the chitosan solution in an organic acid was mixed with an equal volume of 0.lM IICI. After incubating for 3 hours, the viscosity was measured in the same way as that already described.

Statistical analysis. All data are expressed as the means +/- SE (n=8-10). Initially, the homogeneity of variance among 5 groups was evaluated by using Bartlett's test. Significant differences among 5 groups were then evaluated by Newman-Keuls test after one-way ANOVA in the ease of homogenous variance, and by Dunnett's test after the Kruskal-Wallis test in the case of heterogeneous variance.

Results

As shown in the figure, the body weight gain of the control group steadily increased, but that of the chitosan-receiving groups was a little retarded in the early stage of the experimental period regardless of the absence or presence of a supplemental organic acid. From the 4th day, the growth rate of the chitosan-fed groups gradually returned that of the control group, although the chitosan-fed groups could not completely recover the weight initially not gained.

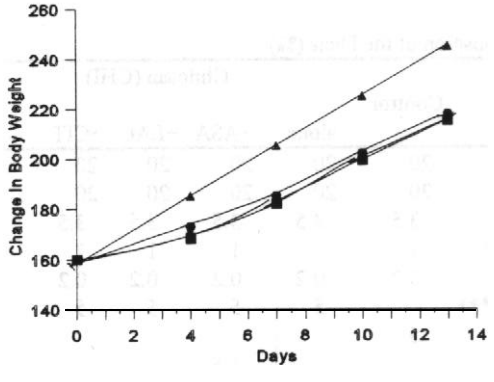

Fig. Change in the Body Weight of Rats Fed on a Cellulose (Control), Chitosan (CHI). CHI Supplemented with Ascorbic Acid (CHI + AsA). CHI supplemented with Lactic Acid (CHI + LAC), and CHI Supplemented with Citric Acid (CHI + CIT)

Points show the mean from 8 to 10 rats. Vertical bars represent the standard error of the mean. Symbols: ▲ control; 0 CHI; △ CHI-AsA; . CHI-CIT; □ CHI-CIT.

Table II indicates the daily average food intake throughout the experimental period, the amount of feces excreted per 3 days (on a dry weight basis) and the lipid content in the feces. Food intake was significantly lower in the CHI + CIT group than in the control group, but the CHI, CHI + AsA and CHI + LAC groups did not differ from the control group. The feces from the CHI and CHI ÷ AsA groups were significantly higher in weight relative to those from the control group.

Interestingly, the fecal weight from the CHI +/- AsA group was about 1.5 times that from the CHI group. The lipid contents in the feces of both the CHI and CHI+ AsA groups were also much higher than those of the control group. Above all, the CHI + AsA group had the highest lipid content in the feces; it was about 5 times higher than that of the control group, and twice that of the CHI, CHI + LAC, and CHI + CIT groups.

The apparent digestibility of fat and protein is shown in Table III. With respect to protein digestibility, the CHI + AsA group was slightly inferior to the control group, but not significantly differing from the other chitosan-fed groups. However, the fat digestibility was significantly reduced by ingesting chitosan, that of the CHI + AsA group being significantly lower than that of the CHI group.

Table II. Food Intake, Fecal Dry Weight and Fecal Lipid Content*

	Food intake (g/day)	Fecal dry wt. (g/rat/3ds)	Fecal lipids (mg/g)
Control	15.7+/-*22.1 [b]	4.13+/-0.569'	115.2+/- 5.6
CHI	13.2+/-0.4[b]	8.329+/-0.563 [b]	492.0+/-28.7 [b]
+AsA	14.8+/-0.9*	12.70+/-0.942'	555.3+/-18.6 [b]
+LAC	13.0+/-0.4*	6.773+/-0.684*	424.8+/-50.4*
+CIT	12.5+/-0.5'	7.168+/-0.947'	408.6+/-46.9*

* Values are not the means +/- SE (n=8-10)
[abc] Means not sharing a comon subscript letter within the same column are significantly different ($p<0.05$).

Table III Comparison between Apparent Fat and Protein Digestibility*

	Digestibility (%)	
	Fat	Protein
Control	94.9+/-.3	95.4+/-0.4*
CHI	52.1+/-4.5[b]	92.7+/-0.9'
+ASA	20.8+/-1.6"	90.7+/-0.7[b]
+LAC	54.4+/-8.7	93.6+/-1.4*
+CIT	58.2+/-8.2[b]	93.4+/-0.9

* Values are the means +/- SD (n-8=10).
[abc] Means not sharing a common subscript letter within the same column are significantly different ($p<0.05$).

Table IV. Fatty Acid Composition of Fecal Lipids

	16:0	18:0	18:1	13:2
Control	139.0+/-40.4	36.4+/-8.3	229.8+/-95.3	468.0+/-236.1
CHI	754.6+/-98.3	198.8+/-22.7	1248+/-228.4	1570+/-320.2
+AsA,	1382+/-l23.5	314.0+/-23.2	2678+/-228.0	3623+/-569.0
+LAC	709.2+/-100.9	191.8+/-22.6	1055+/-220.3	1359+/-t324.7
+CIT	643.5+/-118.5	170.8+/-26.3	1046+/-280.8	1337+/-440.8

• Values are the means +/-SL (n=8-10)
[abc] Means are not sharing a common subscript letter within the same columns are significantly different ($p<0.05$)

Table V. Comparison between Epididymal Fat and Liver Wet Weight*

	Epididymal fat	Liver
Control	1.458+/-0.155	3.15+/-0.11
CHI	0.788+/-0.037 [b]	3.06+/-0.06
+AsA	0.655+/-0.048 [b]	2.97+/-0.06
+LAC	0.669+/-0.073 [b]	3.27+/-0.16
+CIT	0.786+/-0.059 [b]	3.06+/-0.09

* Values are mean +/- SE (n=8-10)
[ab] Means not sharing a common superscript letter within the same column are significantly different ($p<0.05$)

Table VI. Change in Plasma Triaclyglycerol Concentrations

	Day 0	Day 2	Day 4	Day 10
		(mg/dl)		
Control	62.3+/-6.7	89.8+/-8.5	128.1+/-22.0"	167.3+/-29.0
CHI	62.2+/-5.7	49.9+/-4.8	59.2+/-5.7	45.9+/-3.6[b]
+AsA	63.6+/-4.9	43 .6+/-2.4[b]	55.2+/-5.4[b]	39.5+/-4.4[b]
+LAC	65.9+/-6.7	53.7+/-5.9	53.8+/-7.1[b]	60.0+/-9.8
+CIT	64.+/-4.5	57.0+/-17.9	49.8+/-5.1[b]	50.0+/-8.7[b]

* Values are the means +/- SE (n=8-10)
[abc] Means not sharing a common superscript letter within the same column are significantly different ($p<\sim0.05$)

Table VII. Viscosity and Solubility of 0.5%. Chitosan is an Acidic Solution

	Viscosity (CPs) at 37°C		Solubility in an equimolar mixture of HCI and organic acid
	In each solution"	Mixed acid solution*'	
0.lM HCI	40.0	40.0	Completely soluble
0.lM Ascorbic acid	68.5	26.8	Completely soluble
0.1 M Lactic acid	80.4	42.9	Completely soluble
0.lM Citric acid	45.7	41.5	Sparingly soluble

[1] Chitosan (1.5g) was dissolved in 300ml of z 0.1 M acidic solution and incubated at 37°C for 3h, before the viscosity was measured.
[2] Each solution was mixed 1:1 with 0.lM HCI (1 l=0.lM HCI:0.lM organic acid solution).

Table IV summarizes the results of measuring the fatty acid composition of fecal lipids from the rats on each diet. The fecal fatty acids were mainly composed of 16:0, l8:0, l8:l, and 18:2 according to the GLC method described in the previous study.+1 A marked increase in the fecal excretion of these fatty acids was observed for the four chitosan-receiving groups when compared to the control group. Notably, the fecal excretion of 18:2, the major fatty acid in corn oil, was much more increased in the CHI + AsA group than in both the control group in the CHI group. The composition of fatty acids in the chitosan groups was very similar to that of corn oil.

Table V compares the liver and epididymal fat pad weight expressed as a percentage of the body weight. There were no significant differences in the liver weight among all the groups, but in the four chitosan-receiving groups, the epididymal fat pad weight was about half that of the control group, being the lowest in the CHI + AsA group.

Table VI shows the time-course of plasma TG changes throughout the experimental period. The plasma TG level increased with feeding period in the control group, but not in the chitosan receiving groups. The addition of ascorbic acid to chitosan tended to lower the plasma TG level still more, although not significantly.

The viscosity of chitosan in some kinds of organic acids, as well as in their equimolar mixture with HCI, was measured, the results being -shown in Table VII. Chitosan was partially dissolved in 200 volumes of 0. lm citric acid, but 0. lm ascorbic acid and lactic acid dissolved it completely, resulting in quite high viscosity. When a 1% chitosan solution in 0.1 m ascorbic acid was diluted with an equal volume of 0.lm HCI, the viscosity was reduced to less than half its initial value.

Discussion

Chitosan has many characteristic properties that are not found in any other dietary fibers. Some of noticeable properties of chitosan are its bile acid-binding capacity, oil-retaining capacity, and high solubility in acidic media. We have previously reported that chitosan increased the fecal lipid excretion and thereby decreased the apparent fat digestibility to a considerable extent. Some dietary fibers have been reported to inhibit lipid absorption. However, this was considered due to the finding that dietary fiber only delayed and did not impair lipid absorption. It is noteworthy that chitosan is a potent candidate for such a functional fiber.

It is well known that chitosan and cholestyramine have strong bile acid-binding capacity ***in vitro.*** However, the finding that cholestyramine did not increase the fecal lipid excretion ***in vitro*** suggested that its excretory effect could not be simply accounted for by the bile acid-binding capacity.

Moreover, it has been reported that both chitin and chitosan have lipid-retaining capacity, although Knorr has described that chitosan and chitin were inferior and superior to cellulose, respectively, in lipid-binding capacity. For this reason, the ***in vivo*** lipid-excretory effect of chitosan may be interpreted by chemical features other than oil-retaining and -binding capacity.

A noticeable difference between chitosan and other dietary fibers (especially chitin) is its solubility in acidic media. The apparent fat digestibility in the CHI-AsA group was significantly lower than that in the CHI group, although two organic acids (lactic acid and citric acid) did not decrease the apparent fat digestibility compared to chitosan alone. Although lactic acid could dissolve chitosan well in our ***in vitro*** experiment, it could not enhance the inhibition of fat digestibility by chitosan. We thought that the chitosan-caused increase in fecal lipid excretion might not only have depended on the solubility of chitosan in acidic media.

The addition of ascorbic acid to chitosan in an HCI solution markedly decreased the viscosity, suggesting that the inhibition of fat digestion by chitosan was related to the mobility of chitosan in the stomach and to a lowering of the viscosity of chitosan in acidic media. It is to be noted that the supplementation of cellulose with AsA did not affect the apparent fat digestibility (data not shown); in other words, AsA had no effect on fat digestibility.

Accordingly, the mechanism for decreased fat digestibility

by coexisting chitosan and ascorbic acid may be explained as follows: gastric acid-soluble chitosan is mixed with dietary fat in the stomach, the emulsifying process being effectively mediated by ascorbic acid. When the digest emptied from the stomach comes into contact with pancreatic juice (in an alkaline pH range), oil droplets become embedded in the gelled chitosan matrixes and are excreted into the feces without fully undergoing absorption. This is supported by the fatty acid composition of the fecal lipid being very similar to that of corn oil.

In the effect of chitosan on the inhibition of lipid digestion depends only on the foregoing mechanism, it could be seen that chitosan may envelop all nutrients. However, the protein digestibility in rats fed with the chitosan diets was not markedly changed. This result indicates that chitosan mainly affected lipid absorption, and it is necessary to clarity the selectivity in absorption in the future.

In our experiments, chitosan could also affect the growth rate. We thought that this inhibition of growth rate in the early stage of the experiment would depend on the rats not being able to immediately adapt themselves to the diet containing chitosan, and that they could not easily take the diets. We consider that this mainly depended on reducing the energy intake than by the inhibition of lipid digestibility. About 80% of dietary fat could not be digested by supplementing chitosan with ascorbic acid, so the rats could not intake sufficient energy when compared to the control animals.

Despite feeding with a high-fat diet, the plasma TG concentration and epididymal fat pad weight in the chitosan-receiving groups remained at low levels, probably because of a depressed fat intake.

In conclusion, the present study shows that chitosan decreased the apparent fat digestibility, plasma TG levels, epididymal fat pad weight and growth rate of rats fed with a high-fat diet, in which cellulose was replaced by chitosan and that supplementary ascorbic acid augmented the reduction in the apparent fat digestibility of chitosan.

The augmenting effect by ascorbic acid may be explained by a decrease in the viscosity of chitosan and the accompanying progress of mixing chitosan with oil. Chitosan did not affect the apparent protein digestibility, so this effect is considered to be specific to lipids.

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