

Functions of a Chitosan-Orotic Acid Salt in the Gastrointestinal Tract

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A chitosan (CS)–orotic acid salt (CS-OT) was prepared, and the release of orotic acid (OT) from CS-OT as well as the adsorption of bile acids by CS-OT was investigated *in vitro*. The amount of OT released from CS-OT was about 2–2.7 $\mu\text{mol}/\text{mg}$ CS-OT and this changed depending on the species of CS. CS-OT also adsorbed bile acids and the amount increased incrementally according to the number of amino group contained in CS. Furthermore, CS-OT was given to rats as feed in order to investigate the influence on serum cholesterol levels. A decrease in serum cholesterol levels was observed in the group, which was fed a diet containing CS-OT or CS for 1–2 weeks, but no differences in body weight changes were recognized. Therefore, CS-OT may be applied to treating hyperlipidemia.

Key words chitosan; orotic acid; bile acid adsorption; hyperlipidemia

Chitosan (CS) has been used widely in the food industry and is recognized as a dietary fiber that is not absorbed in the gastrointestinal tract because CS is not hydrolyzed by human digestive enzymes. It is generally known that CS potentially interferes with dietary fat absorption and can suppress elevation of plasma cholesterol levels.¹⁾ CS interferes with emulsification of neutral lipids by binding with fatty or bile acids.²⁾ Bile acid is one of the anionic surfactants secreted in the gastrointestinal tract and is formed from cholesterol in the liver. The adsorption of bile acids by polymers in the intestinal tract inhibits their enterohepatic circulation and leads to a decrease in plasma cholesterol levels. Therefore, numerous anion-exchange resins have been synthesized and some have been administered orally as a treatment for hyperlipidemia.³⁾ Because CS is a polysaccharide that possesses polycationic properties, its salts interact with anionic compounds in solution.⁴⁾ CS salts have also been investigated as a vehicle for colon-specific delivery.⁵⁾ We previously reported the uptake of bile acids to alginate gel beads containing CS salts prepared with weak acids, such as lactic acid, and this uptake is the result of complex formation between CS and bile acid.⁶⁾ CS is capable of forming salts with various acids, except mineral acid; *e.g.*, hydrochloric acid or sulfuric acid. And CS salts become useful for oral drug administration if the acid possesses physiological activity, such as orotic acid (OT). OT is a water-soluble vitamin that is responsible for biosynthesis of nucleic acids and is utilized as a component of dietary supplements.⁷⁾ The role of OT in lipid metabolism has recently been studied and the biological functions of OT become clear gradually.⁸⁾ In the present study, CS-OT was prepared, and the release of OT from CS-OT as well as the adsorption of bile acids by CS-OT were investigated *in vitro*. Furthermore, CS-OT was given to rats as feed in an attempt to investigate its influence on serum cholesterol levels.

MATERIALS AND METHODS

Materials Questran (Colestyramine) was kindly supplied by Bristol-Myers Squibb K.K. (Tokyo). One type of chitosan (CS(F), degree of deacetylation; DA, 75–85%) was obtained from Kimitsu Chemical Industries (Tokyo), one type of chitosan (CS1000) was obtained from Wako Chem. Co. (Osaka), and four types of chitosan (7B: DA 70%, 8B:

DA 80%, 9B: DA 90%, 10B: DA 100%) were obtained from Katokichi Co. Ltd. (Tokyo). Chitin (fine powder) was obtained from Kimitsu. OT was obtained from Wako. Alginate, sodium taurocholate (T-CA), glycocholate (G-CA), glycochenodeoxycholate (GC-DA), and taurodeoxycholate (TD-CA) were purchased from Nacalai Tesque (Kyoto). Commercial kits for determination of serum cholesterol and triacylglycerol were obtained from Wako. All chemicals were of reagent grade.

Preparation of CS-OT CS-OT was prepared as follows: CS (0.5 g) was added to 500 ml of 0.072% OT solution and stirred for 1 d at room temperature. It was then filtrated with a glass filter (17G), washed twice with 100 ml ethanol, dried for 8 h on a dish, followed by desiccation in a vacuum in the presence of P_2O_5 . Each size of CS-OT was obtained by sieving with three types of sieve (300, 200, 75 μm).

Determination of OT Released from CS-OT Twenty milligrams of CS-OT was soaked in 0.1 M HCl solution (20 ml) for 2 h at room temperature and the solution was filtered with a membrane filter (Nacalai Tesque; pore size 0.45 μm), and then diluted with 5 mM phosphoric acid. OT content of the solution was determined by HPLC. System comprised an LC-6A pump (Shimadzu, Kyoto), a packed column (Kanto Chem., Tokyo, RP-18 GP 150 mm \times 4.6 mm), and a SPD-6A UV detector (Shimadzu). HPLC was conducted at ambient temperature using an eluent (5 mM phosphoric acid) at a flow rate of 0.8 ml/min and detector wavelength was set at 280 nm.

Adsorption Test of Bile Acid to CS-OT Fifteen milliliters of 2 mM bile acid solution (initial pH 6.0) was placed in an L-shaped tube and maintained at 37 °C. Ten milligrams of CS-OT was added to the solution and shaken at 67 times per min. A 0.2 ml aliquot of each solution was removed periodically and the solution filtered with a membrane filter (0.45 μm) was employed for HPLC analysis.⁹⁾ The amount of bile acid adsorbed by CS-OT was calculated from the difference between the initial amount of bile acid and the residual amount at sampling time. All uptake tests were performed in triplicate.

Animal Study Diets: The powdered feed given to the rats was the certified diet (CRF-1) and was obtained from Oriental Yeast Co. (Japan). The composition of the diet containing cholesterol (HCH-diet) is shown in Table 1. HCH-

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diet containing CS (CS-diet) or CS-OT (CSOT-diet) was prepared by adding 5% CS or 5% CS-OT to the HCH-diet, respectively. HCH-diet containing the physical mixture of CS and OT (PCO-diet) was prepared by adding both 2.9% CS and 2.1% OT to HCH-diet. Each diet was mixed well and given to rats after sifting through a sieve (710 μm).

Experimental protocol was approved by the Ethics Committee at Hokuriku University. Male Wistar rats (5 weeks) were housed individually in stainless-steel wire-bottomed cages in an air-conditioned room, and were allowed free access to food and water for 3 weeks. The food intake and body weight of each rat was measured daily. All rats, except controls (CRF-1 diet group), were fed the HCH-diet for 1 week, followed by each test diet. Blood samples from rats were collected from the superficial lateral caudal vein after 1, 2 and 3 weeks. Blood was clotted at room temperature and centrifuged in an ultracentrifuge (Kokusan H-1300, Japan) at 3000 rpm for 10 min. Serum was separated and total cholesterol and triacylglycerol levels were measured by the enzymatic method and using commercial kits, respectively. If necessary, data were compared using Student's two tailed *t*-test and the difference was considered significant when $p < 0.05$.

RESULTS AND DISCUSSION

When CS-OT is orally administered, it supplies OT to the human body. The amount of OT released from CS-OT prepared with CS(F) was about 2.7 $\mu\text{mol/mg}$ CS-OT, while that released from CS-OT prepared from CS1000 was 2.2 $\mu\text{mol/mg}$ CS-OT. When chitin was treated with OT by same procedure as for the preparation of CS-OT, the product also contained OT. However, the amount of OT was minimal (0.07 $\mu\text{mol/mg}$), and thus the OT contained within CS-OT may be fixed by not by physical adsorption but by ionic bond. These results also show that the amount of OT contained in the CSOT-diet for rats was 21 mg/g.

Questran, a medicine for hyperlipidemia, adsorbed T-CA and the amount of adsorption became constant within 30 min, as shown in Fig. 1. A similar adsorption profile was obtained for CS-OT, and the amount was about 1.5 $\mu\text{mol/mg}$

CS-OT after 30 min, which was 5%/mg CS-OT of the bile acid dissolved in the solution. Figure 2 shows the effects of CS-OT particle diameter on adsorption of T-CA. In all cases, similar adsorption abilities were recognized. The surface of CS-OT appears to be rough and adequate areas for adsorption of bile acid exist on it. Furthermore, hydrated ions may be formed rapidly on the surface and an ion-exchange reaction between OT and bile acid may occur because the adsorption of T-CA is not observed with fine powders of CS (free type) or chitin, which is also capable of a physical adsorption.

Figure 3 shows the effects of deacetylation (DA) of CS on the adsorption of T-CA to CS-OT. The amount of bile acid increased incrementally according to the level of DA in CS.

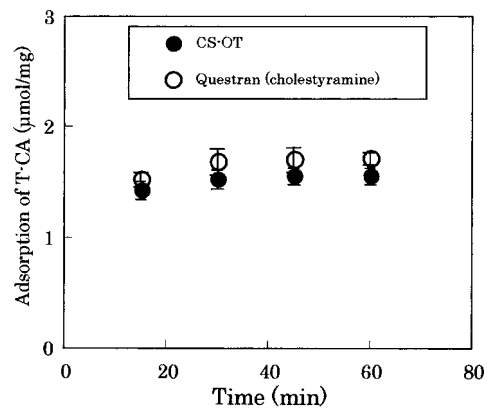


Fig. 1. Adsorption of Taurocholate (T-CA) by CS-OT. Results are expressed as the mean with a bar showing the S.D. of three experiments.

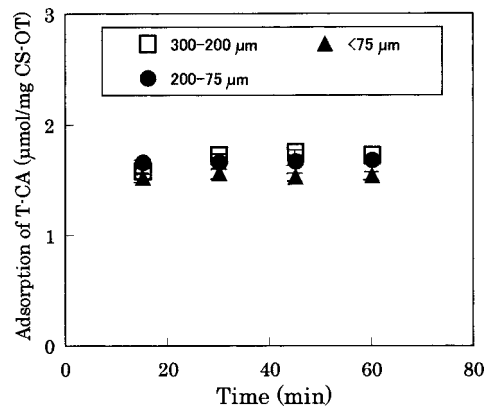


Fig. 2. Effect of CS-OT Diameter on T-CA Adsorption. Results are expressed as the mean with a bar showing the S.D. of three experiments.

Table 1. Composition of HCH-Diet

Component	(%)
CRF-1	96.8
Cholesterol	1.0
Olive oil	2.0
Sodium cholate	0.2

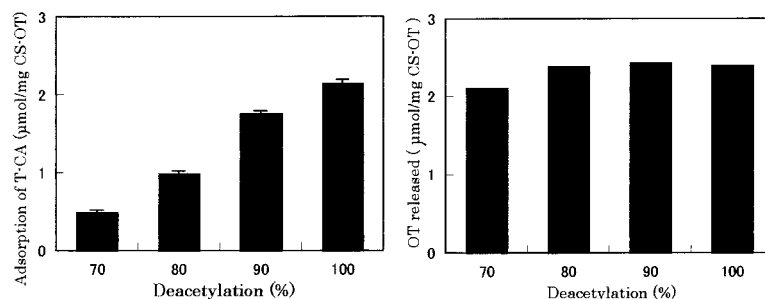


Fig. 3. Effects of Deacetylation of CS on Adsorption of T-CA and Amount of OT Released. Results are expressed as the mean with a bar showing the S.D. of three experiments.

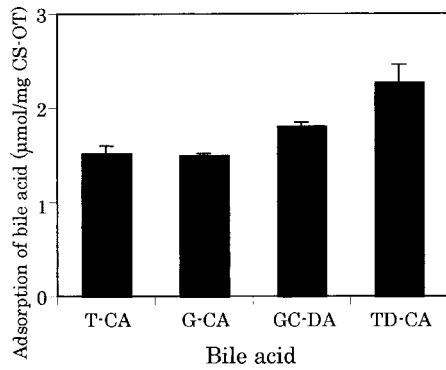


Fig. 4. Adsorption of Bile Acids by CS-OT
T-CA: taurocholate, G-CA: glycocholate, GC-DA: glycochenodeoxycholate, TD-CA: taurodeoxycholate. Results are expressed as the mean with a bar showing the S.D. of three experiments.

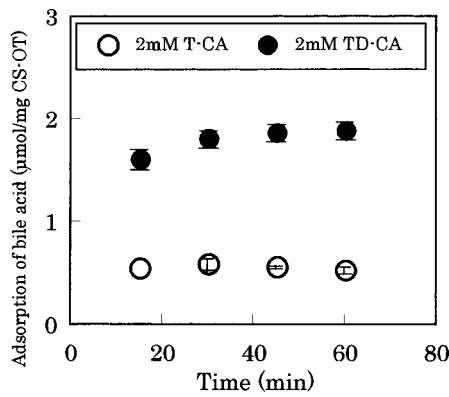


Fig. 5. Adsorption of Bile Acids by CS-OT
Results are expressed as the mean with a bar showing the S.D. of three experiments.

This means that the adsorption is mainly attributable to an ion-exchange reaction between OT and bile acid. The amount of T-CA adsorbed by CS-OT was nearly equal to that of OT released from CS-OT in the case of 10B (DA, 100%). However, in the other cases, all of amino groups contained in CS-OT were not utilized for the adsorption of T-CA. For example, the amount of T-CA adsorbed by 8B was below half of the level adsorbed by 10B. The wettability on the surface of CS salt may change by the adsorption of T-CA because the hydrophilic property of the polysaccharide depends upon the dissociation of the amino group.

The molecular weight of CS did not affect the adsorption ability, although the rates were lower, and the adsorbed amount was $1.00 \pm 0.17 \mu\text{mol/mg CS-OT}$ at 15 min and $1.48 \pm 0.08 \mu\text{mol/mg CS-OT}$ at 30 min with CS1000. As shown in Fig. 4, adsorption of various bile acids was observed. TD-CA, a secondary bile acid was adsorbed particularly efficiently, and the amount adsorbed was $2.27 \pm 0.19 \mu\text{mol/mg CS-OT}$, which is 1.5-fold higher than that of T-CA or G-CA. This phenomenon was remarkably clear when two species of bile acids were present in the test solution. When equal concentrations of T-CA and TD-CA (2 mM) were present, the latter was adsorbed preferentially, and the amount adsorbed by CS-OT was about 3 times that of T-CA, as shown in Fig. 5. This was not observed with a combination of T-CA and G-CA (equal adsorption, $1.1 \mu\text{mol/mg CS-OT}$).

The food intake of rats was not affected by adding chole-

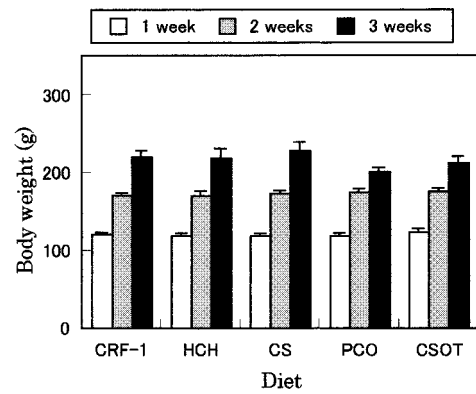


Fig. 6. Changes in Body Weight
Results are expressed as the mean with a bar showing the S.D. of more than three experiments.

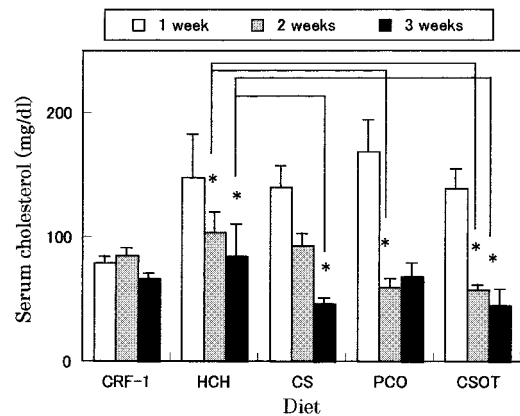


Fig. 7. Changes in Total Serum Cholesterol
Results are expressed as the mean with a bar showing the S.D. of more than three experiments. Significant differences between the two groups, * $p < 0.05$.

sterol, olive oil or sodium cholate to the commercial powder diet (CRF-1). No changes in food intake were observed when CS, CS-OT, or the physical mixture of CS and OT was added to the HCH-diet. As shown in Fig. 6, no differences in body weight changes were seen with regard to the various kinds of feed administered. When rats were fed CRF-1, serum cholesterol levels were $66.7 \pm 3.9 \text{ mg/dl}$ (mean \pm S.D., $n=3$) after 3 weeks. In the case of HCH-diet, cholesterol levels increased to $84.8 \pm 25.4 \text{ mg/dl}$ ($n=9$). However, a pronounced decrease was recognized in rats in some groups, as shown in Fig. 7. In the case of CS-OT diet, mean cholesterol levels were $57.8 \pm 3.7 \text{ mg/dl}$ at 2 weeks and $45.3 \pm 12.4 \text{ mg/dl}$ at 3 weeks, respectively ($n=3$). This phenomenon was also observed with PCO-diet. After 3 weeks, a decrease in serum cholesterol levels was recognized in the CS-diet group. It was recently reported that OT affected by serum cholesterol levels in rat.¹⁰ These data show that CS-OT releases OT by an ion-exchange reaction in the gastrointestinal tract when the CS salt is administered orally.

Serum triacylglycerol levels also decreased in CSOT-diet, PCO-diet and CS-diet groups, as shown in Fig. 8. However, in this study, variation in the level of HCH diet was large and an apparent pharmacodynamic reaction was not seen.

In this study, CS-OT was prepared by a simple method, and the amount of OT released as well as the amount of bile acids adsorbed was investigated. When CS-OT is adminis-

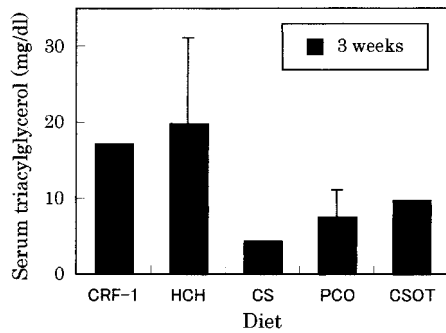


Fig. 8. Effects of Diet on Serum Triacylglycerol

Results are expressed as the mean with a bar showing the S.D. of more than three experiments.

tered orally, it will release OT in the gastrointestinal tract. Even if OT is released from CS-OT in the stomach, which would result in the formation of CS-HCl salt, adsorption of bile acids will still occur.⁹⁾ A decrease in serum cholesterol levels was observed when CS-OT was administered to rats. Consequently, it appears that CS is an anion-exchange resin, and salts, such as CS-OT, may prove to be useful for treating

hyperlipidemia. We are now engaged in work to investigate the influences of the other CS salt administration on serum cholesterol levels.

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