Functions of Chitosan-Ferulic Acid Salt for Prevention of Hypertension

Yoshifumi MURATA^{1*}, Kumiko NAGAKI¹, Kyoko KOFUJI¹ and Toshihiro KISHI²

¹ Faculty of Pharmaceutical Science, Hokuriku University, Ho-3, Kanagawa-machi, Kanazawa 920-1181, Japan

² Nippon Suisan, Ltd., Otemachi, Chiyoda-ku, Tokyo 100-8686, Japan

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A chitosan (CS)-ferulic acid (FA) salt was prepared. FA release from CS-FA and bile acid (BA) adsorption by CS-FA were investigated in vitro. CS-FA released FA into artificial gastric juice or a medium containing BA. Two functions were recognized on alginate or pectin gel beads containing CS-FA: FA release and BA adsorption. Anti-hypertensive effects of CS, FA or CS-FA on hypertensive rats were tested. In rats given a diet containing CS-FA or FA, elevation of systolic blood pressure was suppressed compared to the control diet group, but no difference in food intake or body weight was observed between groups.

Keywords: ferulic acid, chitosan, anti-hypertensive effect, bile acid adsorption, alginate gel bead

Introduction

Polyphenolic substances exist widely in fruits, vegetables and grains. Some of them have been known as antioxidant compounds that show potentially protective effects against hepatic and heart diseases, as well as Alzheimer's disease (Hamaguchi *et al.*, 2009; Rukkumani *et al.*, 2004). A polyphenol, trans-4-hydroxy-3-methoxy-cinnamic acid (ferulic acid, FA), has been used as an anti-oxidizing agent for food-stuffs (Alamed *et al.*, 2009). Its physiological action to mitigate lifestyle-related diseases, such as hypertension, hyperlipidemia, and diabetes, has been noted (Ohnishi *et al.*, 2004; Suzuki *et al.*, 2002; Wang *et al.*, 2004). Oral administration of FA was found to reduce the level of plasma cholesterol and triglyceride in rats (Balasubashini *et al.*, 2003). These results show that intestinal absorption of FA is associated with an H⁺-driven transport system (Zhao *et al.*, 2004). FA has commonly been taken by individuals with the habit of drinking coffee (Renouf *et al.*, 2010).

A natural cationic polysaccharide, chitosan (CS), has also been used in the food industry. It is a dietary fiber that decreases plasma cholesterol by daily uptake (Kaats *et al.*, 2006; Yao *et al.*, 2008). The main mechanisms of the hypocholesterolemic effect is through the adsorption of dietary fat by CS and through adsorption of bile acid (BA) excreted in the intestinal tract (Baker *et al.*, 2009; Shields *et al.*, 2003). In a mouse study, the low bile acid-binding capabilities of CS

have been shown (Bennekum *et al.*, 2005). We reported that CS salts adsorb BA in vitro, but this phenomenon has not been observed in the case of CS itself (Murata *et al.*, 2006). Therefore, following oral administration of CS, the cationic polysaccharide might turn into HCl salt in the human stomach and subsequently bind BA through an ion-exchange reaction.

Although FA is relatively hydrophilic (Sohn and Oh, 2003), it is a weaker acid with more limited water solubility than either acetic or lactic acid. Nevertheless, FA might induce formation of a CS salt because CS was shown to change the salt in a suspension of 2-(4-chlorophenoxy)-2-methylpropionic acid (Murata *et al.*, 2004). Moreover, the electrostatic complex formed between FA and CS (i.e., CS-FA) might be able to induce two functions, FA release and BA adsorption without changing the CS-HCl salt by gastric juice.

In the present study, we attempted to prepare CS-FA and investigated FA release from CS-FA and BA adsorption by the salt *in vitro*. FA released in guts might be absorbed and exhibit the pharmacological action if CS-FA is administered orally for subsequent transfer to the gastrointestinal tract. Therefore, CS-FA was introduced into the feed of spontaneously hypertensive rats (SHR) to investigate its anti-hypertensive effect. In addition, as CS-FA is a fine powder making it difficult to swallow, we devised a form of the salt for swallowing, by incorporating it into calcium-induced alginate gel beads (Alg-Ca) or pectin gel beads. The capabilities of these forms to release FA and absorb BA were examined.

*To whom correspondence should be addressed.

E-mail: y-murata@hokuriku-u.ac.jp

Materials and Methods

For use in this study, FA was purchased from Tsuno Food Ind. Co. Ltd. (Wakayama, Japan). CS (fine powder, degree of deacetylation: 75–85%) was obtained from Kimitsu Chemical Industries Co. Ltd. (Tokyo, Japan) and pectin (GENU pectin LM-102AS) was purchased from Sanshou Co. (Tokyo, Japan). In addition, N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME) was purchased from Nacalai Tesque Inc. (Kyoto, Japan). All other chemicals containing BA were of reagent grade. As an artificial gastric juice, JP XIV 1st medium (pH 1.2) was used.

Preparation of CS-FA CS (1 g), FA (0.5 g) and deionized water (23.5 g) were mixed for 1 day at room temperature. The suspension was then centrifuged at 3000 rpm for 10 min. The precipitate was collected and washed three times with ethanol, dried on a dish, and then desiccated under a vacuum in the presence of P₂O₅. The obtained powder was called CS-FA (2:1). CS-FA (1:1) prepared with CS (1 g) and FA (1 g), and CS-FA (1:2) prepared with CS (1 g) and FA (2 g) were obtained using the same method.

X-ray diffractometry Powder X-ray diffractometry was carried out using an automatic diffractometer (MXP3; MAC Science Ltd., Yokohama, Japan) with a voltage of 40 kV and a current of 20 mA. The scanning rate was 2°/min over a 2 theta range of 5–100°. The results of X-ray diffraction were interpreted using a computer program (Bruker AXS K.K., Yokohama, Japan).

Test of FA release from CS-FA and BA adsorption to CS-FA Either 15 mL of artificial gastric juice, deionized water or a 2 mmol/L BA solution (pH 5.9–6.0) was placed into a vial tube and maintained at 37°C. Then, 15 mg of CS-FA was added to the tubes and shaken. After 2 h, the FA released from CS-FA or BA adsorbed to CS-FA was measured using HPLC. All tests were performed in triplicate. The HPLC system had an LC-6A pump (Shimadzu Co., Kyoto, Japan), a packed column (150 mm × 4.6 mm, Mightysil RP-18 GP; Kanto Chemical Co., Tokyo, Japan), and a SPD-6A UV detector (Shimadzu Co.). The HPLC for determination of FA was conducted at ambient temperature using an eluent containing 10 mmol/L phosphate buffer (pH 4.7) and methanol (8:2) at a flow rate of 0.8 mL/min. The detector wavelength was set as 260 nm. HPLC for the determination of BA was conducted at ambient temperature using an eluent containing methanol, 30 mmol/L phosphate buffer (pH 3.4), and acetonitrile (6:3:1) at a flow rate of 0.8 mL/min. The detector wavelength was set as 230 nm. The amount of BA adsorbed to CS-FA was calculated based on the amount of BA added and that remaining at sampling time.

Preparation of gel beads containing CS-FA

We prepared Alg-Ca (hydrogel beads) containing CS-

Table 1. Composition of test diets.

Diet	Components (%)			
	CRF-1	CS-FA(2:1)	CS	FA
Control	100	0	0	0
CS-FA	90	10	0	0
CS	92.1	0	7.9	0
FA	97.9	0	0	2.1

FA as follows: 1 g of CS was dispersed in 9 g of 1(w/w)% sodium alginate solution with agitation. This solution (2 g) was dropped into 10 mL of 0.1 mol/L CaCl₂, then left to stand at room temperature for 1 h. The spherical beads were transferred to 10 mL of distilled deionized water containing 0.1 g of FA, which was incubated at 37°C for a day. Pectin gel beads containing CS-FA was also prepared with 2(w/w)% pectin instead of sodium alginate using the method described above. Dried gel beads were obtained by drying the hydrogel at for 8 h on a dish, followed by vacuum treatment in a desiccator in the presence of P₂O₅.

Test of FA release and BA adsorption by gel beads containing CS-FA Gel beads containing CS-FA corresponding to 2 g of hydrogel were added to 15 mL of 2 mmol/L BA solution incubated at 37°C. A 0.2-mL aliquot of the solution was removed periodically to determine FA released and BA taken up into the gel beads using HPLC as described.

Animal study The powdered feed given to the rats was a certified diet (CRF-1; Oriental Yeast Co. Ltd., Japan). The composition of the diet containing CS, FA or CS-FA (2:1) is shown in Table 1. Each ration was mixed well and given to rats after sifting through a sieve (710 µm).

The Animal Care and Use Committee of Hokuriku University approved the experimental protocol. Male SHR/Izm rats (4-weeks-old, 60–100 g; Sankyo Lab.) were housed individually in stainless-steel wire-bottomed cages in a temperature-controlled room. They were allowed free access to food and water for 1 week. For the following 5 weeks, 0.003% L-NAME was used as drinking water (Pechanov *et al.*, 1999). The food intake of each rat was measured daily and body weight was measured weekly. Systolic blood pressure (SBP) was measured weekly using the tail-cuff method in conscious SHR placed in a holder using an automatic blood pressure monitoring system (MK-2000; Muromachi Kikai Co. Ltd., Japan). Where necessary, data were compared using ANOVA (Tukey's test) and their difference was inferred as significant when $p < 0.01$.

Results and Discussion

Figure 1 shows the X-ray diffraction patterns obtained from CS-FA, CS, FA and their physical mixtures; FA exhibited a characteristic crystalline compound pattern of dif-

Fig. 1. X-ray diffractograms.

[A] CS, [B] CS-FA(2:1), [C] CS-FA(1:1), [D] CS-FA(1:2), [E] CS treated with deionized water, [F] FA, [G] mixture of FA and CS (1:10), [H] mixture of FA and CS (1:5), [I] mixture of FA and CS (1:2), [J] mixture of FA and CS (1:1).

fraction. The pattern was also apparent in the mixture of CS and FA. On the other hand, CS-FA showed a pattern which lacked the characteristic diffraction peaks of FA. This result shows that the crystal form of FA is only slightly present in CS-FA. The broad peak of CS at 20° decreased according to the increment of FA amount in the CS-FA preparation. The peak disappearance was not recognized in the case of CS treated with deionized water and not contain FA or in the case of the physical mixtures.

CS-FA released FA into artificial gastric juice: 0.434 ± 0.006 mmol/g CS-FA(2:1); the amount was 0.058 ± 0.002 mmol/g CS-FA(2:1) in deionized water. This result indicates that FA mainly exists as an ion pair with the amino group of CS in CS-FA. The FA is liberated from CS-FA when hydrogen chloride is exchanged for FA on the polymer. The CS-FA also released FA into the medium containing BA as it simultaneously adsorbed BA as shown in Figure 2. In the case of taurocholate (TCA), the amount of FA released was 0.261 ± 0.001 mmol/g CS-FA(2:1) and the amount of TCA adsorbed was 0.280 ± 0.002 mmol/g CS-FA(2:1).

A sample of Alg-Ca prepared in this study theoretically contains about 0.1 g of CS, which was converted to CS-FA within the gel matrix by incubation in FA suspension. Two functions of CS-FA were recognized on Alg-Ca containing CS-FA: the release of FA and the adsorption of BA. Figure 3 shows when hydrogel beads (2 g) were shaken in 15 mL of 2 mmol/L TCA solution, it immediately took up TCA (2 h, 24.7 ± 0.2 μ mol). The uptake was observed for other BAs such as glycocholate (25.9 ± 0.2 μ mol), taurodeoxycholate (28.2 ± 0.3 μ mol), and taurochenodeoxycholate (28.1 ± 0.1

Fig. 2. Chromatograms of TCA obtained from HPLC determination.

[A] TCA solution, [B] TCA solution with CS-FA.

Fig. 3. FA release (○) from Alg-Ca containing CS-FA and uptake of TCA (●). Values are means $\pm$ SD of triplicate analyses.

μmol) after 2 h. In all cases, the upper 80% of BA dissolved in the solution was taken up; the uptake amount increased according to the increment of BA concentration (data not shown). On the other hand, the gel beads gradually released FA: the amount released was $59.7 \pm 1.6 \mu\text{mol}$ after 2 h. Similar patterns of FA release and TCA uptake were apparent in the case of dried Alg-Ca. The FA existed not only as CS-FA, but as a free inner gel matrix. Both types were released when Alg-Ca was immersed in a TCA solution.

Therefore, the amount of FA released was greater than that of TCA taken up into Alg-Ca. An identical phenomenon was recognized when pectin beads containing CS-FA were used as the vehicle. The amount of TCA adsorbed after 2 h was $25.5 \pm 0.3 \mu\text{mol/sample}$ and the amount of FA released was $60.2 \pm 4.1 \mu\text{mol/sample}$ in the case of calcium-induced pectin beads.

When administered orally, FA might be released from CS-FA and absorbed in the gastrointestinal tract. Reportedly, FA transport occurs via an H^+ -dependent system in Caco-2 cells (Itagaki *et al.*, 2005). Moreover, supplementation with FA has shown physiological actions like a protective effect on hyperlipidemia in diabetic rats (Zhao *et al.*, 2004). In the present study, CS-FA, FA or CS was mixed into the powder diet given to SHR for 5 weeks. No changes in food intake (about 19 g/d) were observed in any diet group, and no apparent difference in body weight changes resulted from the various administered foods (Figure 4). In the case of the control diet group, SBP was initially $125 \pm 12 \text{ mmHg}$ ($n=5$) and

increased gradually to $263 \pm 13 \text{ mmHg}$ after 5 weeks (Figure 5). In the case of the CS-FA diet group (theoretically about 1.9 g of CS-FA was taken up per day), SBP was initially $126 \pm 8 \text{ mmHg}$ and its elevation was suppressed compared to the control group; it was $188 \pm 12 \text{ mmHg}$ after 5 weeks. FA is known to be beneficial for preventing the elevation of SBP (Suzuki *et al.*, 2002), which was confirmed based on the identical phenomenon being observed with the FA diet group, while no pronounced effect was recognized in rats in the CS diet group. These results show that the effect might be mainly attributed to FA released from CS-FA into the gastrointestinal tract.

Both CS and FA have been used as food additives. CS-FA was prepared using a simple method, with two functions: FA release and BA adsorption. These functions are also expected in the polysaccharide gel matrix, which is suitable for oral administration. As CS-FA could release FA into artificial gastric juice or bile acid solution, FA might be released into the stomach and intestine when CS-FA is administered orally. The FA is known to be absorbed into the gastrointestinal tract and to show a pharmacological action. Actually, elevation of blood pressure in SHR was suppressed by the daily intake not only of FA, but also of CS-FA. On the other hand, CS-FA might change the CS-HCl salt in the stomach, allowing the salt to adsorb BA as well as CS-FA (Ylitalo, *et al.*, 2002).

In the present study, L-NAME was given to SHR as drinking water to generate malignant hypertension (Takahashi, *et al.*, 2007). Development of a preparation that does

Fig. 4. Change in food intake and body weight of SHR/Izm rat ($n=5$).

*Food intake is the average of one week.

Fig. 5. Effect of diet on change in SBP ($n=5$).

* $P < 0.01$, compared with the control.

not impart harmful effects is essential for prevention of lifestyle-related diseases such as hypertension. Therefore, we shall investigate further the preventive effect of CS-FA on such diseases.

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