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Investigation of the cholesterol-lowering action of insoluble fibre derived from the peel of *Citrus sinensis* L. cv. Liucheng

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Abstract

A water-insoluble fibre-rich fraction (WIFF) was isolated from the peel of *Citrus sinensis* L. cv. Liucheng. The effects of a WIFF-containing diet on lipid and cholesterol absorption in hamsters were investigated and compared with those of a cellulose-containing diet and fibre-free diet, as controls. Results demonstrated that WIFF could significantly ($P < 0.05$) decrease the levels of serum triglyceride, serum total cholesterol, liver total lipids, and liver cholesterol, while it could also significantly ($P < 0.05$) increase the levels of fecal total lipids, fecal cholesterol, and fecal bile acids, as well as the fecal bulk and moisture. The pronounced hypocholesterolemic and hypolipidemic effects of WIFF might be attributed to its ability to enhance cholesterol and bile acids excretion. These results suggest that WIFF could be a potential cholesterol-lowering ingredient in human diets or new formulations of fibre-rich functional foods.

Keywords: Insoluble fibre; Hypocholesterolemic effect; Hypolipidemic effect; *Citrus sinensis* L. cv. Liucheng; Peel

1. Introduction

An increased consumption of insoluble as well as soluble dietary fibres has been reported to be effective in lowering the risk of cardiovascular disease, gastrointestinal disease, colon cancer, glycemic response and obesity (Nishimune et al., 1991; Rosamond, 2002; Slavin, 2001). Recent findings (Marlett, 2001; Schieber, Stintzing, & Carle, 2001) have demonstrated that the insoluble fibres derived from some fruits and vegetables may promote a significant decrease in blood cholesterol concentration. The reduction in the levels of cholesterol and lipid by dietary fibres can be a consequence of alternations in dietary intake, reduced cholesterol biosynthesis, change of bile acid synthesis, and reduced absorption of lipid, cholesterol, and bile acid (Lairon, 2001; Marlett, 2001). The importance of dietary fibres in diet therefore leads to a trend to find new sources of fibres as food ingredients.

Citrus sinensis L. cv. Liucheng (Liucheng sweet orange) is an important fruit for juice production. After the juice extraction process, thousands of tons of pomace, in the form of orange peel, are produced and discarded as feed. Our previous study (Chau & Huang, 2003) reveals that the peel of Liucheng sweet orange (LSO) possesses a high level of water-insoluble fibre-rich fraction (WIFF) (50.2 g/100 g of peel) which has distinctive physicochemical properties for food applications. A linear correlation has been observed between the physicochemical properties and the hypocholesterolemic action of insoluble fibres (Chau & Cheung, 1999; Gordon, 1989). Accordingly, it is worth assessing the influence of the peel WIFF on cholesterol absorption via in vivo studies for the purpose of exploiting this fibre-rich fraction as a promising functional food ingredient.

The objective of the present study was to evaluate the potential hypocholesterolemic action of the WIFF derived from the LSO peel on hamsters fed diets supplemented with cholesterol (1.0 g/100 g of diet). The influences of the WIFF on the levels of lipid, cholesterol, and bile acids were investigated, and the relationship

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between the physicochemical properties and the physiological aspects of the WIFF was interpreted.

2. Materials and methods

2.1. Peel samples

After the juice extraction process, the peel sample of *C. sinensis* L. cv. Liucheng was collected from CHIA-MEEI Food Industrial Corp., Taiwan. The peel sample was dried in an air-oven at 40 °C for 48 h, and the moisture content of the dried peel was 7.57 ± 0.04 g/100 g. The dried sample was then finely ground to 0.5 mm in size.

2.2. Separation of water-insoluble fibre-rich fraction (WIFF)

According to the method of Chau and Huang (2003), WIFF was prepared by homogenising the LSO peel sample in cold distilled water (1:10, w/v), using the Osterizer (Sunbeam-Oster, Chicago, IL) at the "Hi" speed for 2 min. After filtration, WIFF was washed with water and 85% ethanol, and then dried by solvent exchange and air at 30 °C. In this study, small amount of impurities, such as protein and ash (3.92 and 3.08 g/100 g of WIFF, respectively), were found in the WIFF obtained.

2.3. Diet preparation

Three experimental diets, named as fibre-free, cellulose, and WIFF diets, were prepared according to the formulations of the AIN93M diet (Reeves, Nielsen, & Fahey, 1993) with slight modifications (Table 1). Cellulose (ICN Nutritional Biochemicals, Cleveland, OH) and WIFF were added as the sole source of fibre in the cellulose and WIFF diets, respectively. In the fibre-free diet, additional corn starch was added instead of the fibre. All three diets were supplemented with 1% (w/w) cholesterol to induce an alimentary hypercholesterolemia in the hamsters.

Table 1
Formulations of the test diets

Ingredients[a]	Fibre-free diet	Cellulose diet	WIFF diet
Casein	14.0	14.0	13.8
WIFF[b]	–	–	5.38
Cellulose	–	5.00	–
Sucrose	10.0	10.0	10.0
Corn starch	66.1	61.1	60.9
Soybean oil	4.00	4.00	4.00
Choline bitartrate	0.25	0.25	0.25
L-Cystine	0.18	0.18	0.18
AIN-93M vitamin mix	1.00	1.00	1.00
AIN-93M mineral mix	3.50	3.50	3.50
Cholesterol	1.00	1.00	1.00

[a] The ingredients are expressed as g/100 g of diet (dry weight). Casein, cellulose, choline bitartate, L-cystine, AIN-93M vitamin mix, AIN-93M mineral mix, and cholesterol is obtained from ICN Nutritional Biochemicals (Cleveland, OH).

[b] WIFF contained protein and ash at a level of 3.92 and 3.08 g/100 g of WIFF, respectively.

2.4. Experimental design

Twenty-four male Golden Syrian hamsters (6 weeks old), weighing 86.7 ± 6.1 g were obtained from the National Laboratory Animal Center of Taiwan. After a seven-day acclimitisation period, the hamsters were randomly divided into three diet groups of eight animals each, and were housed (in pairs) in stainless steel screen-bottomed cages in a room maintained at 24 ± 1 °C with a 12 h light:dark cycle. During the experimental period (30 days), hypercholesterolemic diets and water were provided with ad libitum access. Food intakes and animal weighs were recorded every 48 h, and feces were collected and weighed daily. At the end of the experimental period, the hamsters were sacrificed after fasting for 12 h. Blood was drawn from the animals by cardiac puncture, and serum was prepared for biochemical tests. Different organs were removed, weighed, and kept at −70 °C for analysis. Feces were lyophilised, weighed, ground, and stored at −20 °C until analysed.

2.5. Determination of serum cholesterol and lipid

Using commercially available assay kits, concentrations of total cholesterol (No. 402, Sigma Chemical Co., St. Louis, MO), high-density lipoprotein (HDL) cholesterol (No. 352, Sigma Chemical Co.) and triglyceride (Merckotest 14354, Merck, Germany), in the serum samples, were enzymatically determined.

2.6. Determination of liver cholesterol and lipids

Total liver lipids were extracted from 1–2 g of liver with chloroform:methanol mixture (2:1, v/v) (Folch, Lees, & Stanley, 1957). Liver cholesterol in the liver lipids extract was then determined colorimetrically at 490 nm (Searcy & Bergquist, 1960). The content of total lipids in the liver was quantified gravimetrically by evaporating off the solvents in the liver lipids extract.

2.7. Determination of fecal cholesterol and lipids

According to the method of Folch et al. (1957), the fecal cholesterol and total lipids in the dried feces samples were extracted with chloroform:methanol (2:1, v/v) mixture. The content of fecal cholesterol in the fecal lipids extract was quantified by the method of Searcy and Bergquist (1960). Furthermore, the fecal total lipids were determined gravimetrically by evaporating off the organic solvent in the liver lipids extract.

2.8. Determination of fecal bile acids

According to the method of Beher, Stradnieks, Lin, and Sanfield (1981), fecal bile acids in the fecal samples collected on the last 3 days of the experiment were extracted by boiling ethanol. Using the method of Turley and Dietschy (1978), the content of fecal bile acids in the bile acid extract was determined as follows: a reaction mixture that contained 750 μl of Tris–HCl buffer (0.133 M Tris, 0.666 mM EDTA, pH 9.5), 500 μl of 1 M hydrazine hydrate (pH 9.5), 150 μl of 7 mM NAD^+ (N-7004, Sigma Chemical Co.) and 50 μl of bile acid extract or standard (T-0750, Sigma Chemical Co.) was pre-incubated at 30 °C before the addition of 100 μl of hydroxysteroid dehydrogenase (HSD) (H-8879, Sigma Chemical Co.) dissolved in 0.03 M Tris–HCl buffer. After incubation at 30 °C for further 60 min, the absorbance of the mixture was determined at 340 nm against reagent blank, in which the HSD solution was replaced by Tris–HCl buffer.

2.9. Statistical analysis

All determinations, which were carried out in triplicate, were analysed by one-way analysis of variance, using the Statistical Analysis System (SAS). An α level of 0.05 was set to determine statistical significance.

3. Results and discussion

Composition of the test diets is shown in Table 1. The amount of fibre in each diet was set at a level of 5%. Because of the presence of small amounts of protein and ash in the WIFF (3.92 and 3.08 g/100 g of WIFF, respectively), the exact amount of WIFF added was 5.38 g/100 g of diet. Table 2 summarises the food intake, body weight gain, and organ weights of hamsters fed the fibre-free, cellulose, and WIFF diets. On the basis of daily observations, all animals remained healthy and active throughout the experiment. After 30 days of feeding, the body weights of the hamsters increased from 83.6–88.5 g (initial weight) to 109–115 g (final weight) among the three diet groups. The results showed that the experimental diets, with or without the inclusion of fibre, did not interfere with the food intake (7.04–7.98 g/day) body weight gain (0.79–0.91 g/day). In Table 2, the weights of visceral organs, such as kidney, cecum, small intestine, colon, and rectum, are normalised to kilogramme body weight of the hamsters. There were no significant differences in the weights of visceral organs among the hamsters fed the three diets over the experimental period. This suggested that the consumption of WIFF as well as cellulose did not affect the weights of visceral organs.

The levels of serum triglyceride, total cholesterol, and HDL cholesterol of the hamsters fed the WIFF, cellulose, and fibre-free diets are listed in Table 3. Compared to the fibre-free diet group, WIFF could significantly ($P < 0.05$) decrease the level of serum triglyceride by 45% while cellulose gave only a slight reduction in the serum triglyceride level. Some recent studies (Davignon & Cohn, 1996) have indicated that decreased plasma triglyceride concentration was associated with a lower risk of coronary heart disease. The reduction in triglyceride level due to dietary fibre may be a result of the direct interference of triglyceride absorption as well as increased excretion of triglyceride via fecal fat (Miettinen, 1987).

Compared to the fibre-free diet (264 mg/dl), the consumption of cellulose and WIFF diets could significantly ($P < 0.05$) lower the serum total cholesterol level by 30.3% and 47.0%, respectively (Table 3). This showed that the inclusion of insoluble fibres such as WIFF and cellulose in diets could effectively decrease the serum total cholesterol concentration, with the hypocholesterolemic effect of WIFF being much greater than that of cellulose. The fibres derived from grape, persimmon, and some other agricultural byproducts (e.g., sugar beet pulp and apple pomace) may also possess hypocholesterolemic and hypolipidemic properties, and different fibres have significantly different effects on plasma lipids (Gorinstein, Bartnikowska, Kulasek, Zemser, & Trakhtenberg, 1998; Leontowicz, Gorinstein, Bartnikowska, Leontowicz, Kulasek, & Trakhtenberg, 2001; Martin-Carron, Goni, Larrauri, Garcia-alonso, & Saura-Calixto, 1999). However, it should be pointed out that the hypocholesterolemic effects of WIFF might not hold true when the particular WIFF being tested is added at a level of less than 5% in the hypercholesterolemic diet.

Table 2
Effects of water-insoluble fibres on food intake,[a] weight gain,[a] and organ weight of hamsters

Diets	Food intake (g/day)	Weight gain (g/day)	Organ weight[a,b]			
			Kidney	Cecal wall	Small intestine	Colon and rectum
Fibre-free	$7.04 \pm 0.10x$	$0.81 \pm 0.09x$	$0.91 \pm 0.10x$	$0.74 \pm 0.14x$	$1.83 \pm 0.21x$	$2.00 \pm 0.41x$
Cellulose	$7.98 \pm 0.24x$	$0.79 \pm 0.28x$	$0.78 \pm 0.07y$	$0.72 \pm 0.10x$	$1.79 \pm 0.13xy$	$2.31 \pm 0.61x$
WIFF	$7.29 \pm 0.20x$	$0.91 \pm 0.16x$	$0.73 \pm 0.04y$	$0.68 \pm 0.13x$	$1.61 \pm 0.11y$	$1.90 \pm 0.43x$

[a] Values (means ± standard deviation) in the same column with different letters are significantly different (Duncan, $P < 0.05$).
[b] Organ weight was expressed as g/100 g of body weight on a wet weight basis.

Table 3
Effects of water-insoluble fibres on serum triglyceride and cholesterol contents[a] of hamsters

Diets	Triglyceride (mg/dl)	Total cholesterol (mg/dl)	HDL cholesterol (mg/dl)	HDL:total cholesterol ratio[a]
Fibre-free	$175 \pm 19.1x$	$264 \pm 22.9x$	$120 \pm 17.0x$	$0.46 \pm 0.13x$
Cellulose	$150 \pm 1.84x$	$184 \pm 24.3y$	$137 \pm 12.6y$	$0.74 \pm 0.08y$
WIFF	$96.2 \pm 22.9y$	$140 \pm 19.5z$	$102 \pm 17.0x$	$0.73 \pm 015y$

[a] Values (means ± standard deviation) in the same column with different letters are significantly different (Duncan, $P < 0.05$).

Table 3 reveals that the consumption of cellulose diet versus fibre-free diet could lead to an increase in the HDL cholesterol level of 14%, while there were no significant differences in the HDL cholesterol levels between the WIFF and fibre-free diet groups (102–120 mg/dl). Some previous studies (Uberoi, Vadhera, & Soni, 1992) have indicated that the lowering of total cholesterol might be mainly associated with the LDL and VLDL cholesterol fractions. In the present study, the 45% reduction in triglyceride level (Table 3) was perhaps attributable to the reduced absorption of triglyceride, as well as the lower concentrations of VLDL and LDL, leading to less of the circulating form of triglyceride.

As shown in Table 3, the reduction in serum total cholesterol with the WIFF and cellulose diets was accompanied by a significantly ($P < 0.05$) higher HDL:total cholesterol ratio (0.73–0.74) as compared to the fibre-free diet (0.46). As the HDL:total cholesterol ratio may be negatively correlated with the risk of coronary heart disease, even when total cholesterol level is elevated (Barter & Rye, 1996; Malaspina, Bussiere, & Calve, 1981), the high proportion of HDL cholesterol (73% of total cholesterol) in hamsters fed the WIFF diet suggests the anti-atherogenic potential of WIFF.

There were no significant differences in the liver weight and liver:body weight ratio among the hamsters fed the three diets (Table 4). Variations in the appearance and weight of the livers are generally related to the amount of cholesterol and oil incorporated in the experimental diets (Beynen, Lemmens, Bruije, Katan, & Nab Zutphen, 1986). As the fibre source was the only variable in the ingredients of the test diets (Table 1), the liver weights among the hamsters were therefore comparable to each other. Table 4 shows that the level of liver total lipids obtained with the WIFF diet (16.8 g/100 g of liver) was significantly lower ($P < 0.05$) than those with the cellulose and fibre-free diets (18.9 and 19.1 g/100 g of liver, respectively) which were comparable to each other. Moreover, both the WIFF and cellulose diets significantly ($P < 0.05$) decreased the liver cholesterol level (5.11–6.06 g/100 g of liver) by 38.5% and 27.1%, respectively, as compared to the fibre-free diet (8.31 g/100 g of liver) (Table 4). These results demonstrated that WIFF was statistically more effective in lowering the levels of cholesterol and lipids of the hepatic tissues than cellulose. Some authors have demonstrated that the fibres derived from pulses, cereals, persimmon, and some agricultural byproducts, such as sugar beet pulp and apple pomace, could significantly decrease the concentration of liver cholesterol and lipids, to different extents (Gorinstein et al., 1998; Leontowicz et al., 2001; Uberoi et al., 1992). Among the three diet groups, the trend of reduction in the levels of liver total lipids and cholesterol (Table 4) paralleled those of serum triglyceride and total cholesterol (Table 3). It was speculated that the decrease in the serum cholesterol level with the WIFF diet was at least in part due to the reduced hepatic synthesis of cholesterol.

The stool moisture content, fecal dry weight, and concentrations of total lipids, cholesterol, and bile acids in the feces of hamsters fed the WIFF, cellulose, and fibre-free diets are presented in Table 5. The addition of WIFF and cellulose, providing 5 g of insoluble fibre per 100 g of diet, significantly ($P < 0.05$) increased the stool moisture content by 53% and 24%, respectively, compared to that with the fibre-free diet (13.4 g/100 g of feces). Based on our previous findings on the water-holding capacity (16.7 and 3.81 ml/g, respectively) and swelling property (16.7 and 4.97 ml/g, respectively) of WIFF and cellulose (Chau & Huang, 2003), high correlations between the stool moisture content and water-holding capacity ($r = 0.97$), as well as the stool moisture content and swelling property ($r = 0.99$), were observed. Fecal dry weights with the WIFF and cellulose diets (1.35 and 1.36 g/day, respectively) were comparable to each other, and were significantly ($P < 0.05$) increased by 55–56% relative to the fibre-free diet (0.87 g/day) (Table 5). These results proved that the consumption of insoluble fibre could significantly increase the fecal weight, as well as fecal bulk. Similarly, fibres derived

Table 4
Effects of water-insoluble fibres on liver weight,[a] liver total lipids,[a] and liver cholesterol[a] of hamsters

Diets	Liver weight (g)	Liver:body weight ratio[a]	Liver total lipids (g/100 g of liver)	Liver cholesterol (g/100 g of liver)
Fibre-free	$5.76 \pm 0.67x$	$0.06 \pm 0.00x$	$19.1 \pm 1.37x$	$8.31 \pm 1.43x$
Cellulose	$5.79 \pm 0.48x$	$0.05 \pm 0.00x$	$18.9 \pm 1.35x$	$6.06 \pm 0.37y$
WIFF	$6.24 \pm 0.54x$	$0.05 \pm 0.00x$	$16.8 \pm 1.36y$	$5.11 \pm 0.31z$

[a] Values (means ± standard derivation) in the same column with different superscripts are significantly different (Duncan, $P < 0.05$).

Table 5
Effects of water-insoluble fibres on stool moisture content,[a] fecal dry weight,[a] fecal total lipids,[a] fecal cholesterol,[a] and fecal bile acids[a] of hamsters

Diets	Stool moisture content (g/100 g of feces)	Fecal dry weight (g/day)	Fecal total lipids (mg/day)	Fecal cholesterol (μmol/day)	Fecal bile acids (μmol/day)
Fibre-free	$13.4 \pm 0.29x$	$0.87 \pm 0.11x$	$0.06 \pm 0.01x$	$65.9 \pm 11.7x$	$24.0 \pm 3.63x$
Cellulose	$16.6 \pm 1.16y$	$1.36 \pm 0.15y$	$0.07 \pm 0.00y$	$89.8 \pm 6.96y$	$51.7 \pm 18.2y$
WIFF	$20.5 \pm 0.84z$	$1.35 \pm 0.42y$	$0.08 \pm 0.01z$	$106 \pm 12.0z$	$66.0 \pm 6.72y$

[a] Values (means ± standard deviation) in the same column with different superscripts are significantly different (Duncan, $P < 0.05$).

from apple pulp, sugar-beet, and vegetables, such as rutabagas, carrots, green beans, Brussels sprouts, and peas were also found to increase fecal dry weight effectively (Bravo, Saura-Calixto, & Goni, 1992; Nyman, Schweizer, Tyren, Reimann, & Asp, 1990). The increase in fecal weight might vary widely with the type and quantity of dietary fibre being consumed (Shankardass et al., 1990).

As compared with the cellulose and fibre-free diets, the consumption of WIFF diet could significantly ($P < 0.05$) increase the levels of fecal total lipids (14% and 33%, respectively), fecal cholesterol (18% and 61%, respectively), and fecal bile acids (28% and 175%, respectively) (Table 5). In Tables 3–5, negative relationships were observed between the levels of fecal bile acids and serum total cholesterol, as well as liver cholesterol, suggesting that the reduction in levels of serum and liver cholesterol was partially associated with the increased excretion of fecal bile acids. Increased excretion of fecal lipids and sterol was also observed with the consumption of pectin, wheat bran, oat bran, and some other cereal fibres (Lairon, 2001; Marlett, 2001). The ability of fibres to bind bile acids may play a role in lowering serum cholesterol by preventing their reentry into circulation and eventual losts in excretion (Hughes, 1991; Normand, Ory, & Mod, 1987).

Our results show that the decreases in serum triglyceride and total cholesterol (Table 3), as well as liver total lipids and cholesterol (Table 4), were probably due to the promoted excretion of cholesterol, lipid, and bile acids in stools (Table 5). These results could be supported by the negative relationships found between the fecal cholesterol and serum cholesterol, fecal cholesterol and liver cholesterol, fecal total lipids and serum triglyceride, and fecal total lipids and liver total lipids (Tables 3–5). In general, many effects of dietary fibres, such as decreased transit time, higher bile acid adsorption, increased cholesterol catabolism to bile acids, retarded cholesterol biosynthesis, and decreased cholesterol absorption, could lead to a greater excretion of fecal sterol, and subsequently decrease the serum cholesterol level (Hughes, 1991; Lairon, 2001; Marlett, 2001; Uberoi et al., 1992). Furthermore, the enhanced excretion of fecal total lipids and cholesterol by WIFF might be in part due to its high cation-exchange capacity (523 mequiv/kg) relative to cellulose (23.0 mequiv/kg) (Chau & Huang, 2003). Insoluble fibres with high cation-exchange capacity might destabilise, entrap, and disintegrate the micelles and hinder the diffusion or absorption of micelles, leading to the reduction in absorption of lipids and cholesterol (Furda, 1990).

4. Conclusions

The present study demonstrates that the WIFF derived from the LSO peel had very pronounced hypocholesterolemic and hypolipidemic effects as compared to cellulose. It could significantly ($P < 0.05$) decrease the levels of serum triglyceride, serum total cholesterol, liver total lipids, and liver cholesterol, while it also significantly ($P < 0.05$) increased the levels of fecal total lipids, fecal cholesterol, and fecal bile acids as well as the fecal bulk and moisture. These results suggest that the hypocholesterolemic action of WIFF is due to its ability to enhance cholesterol and bile acids excretion. WIFF could be a potential cholesterol-lowering ingredient in human diets, and offer industry an opportunity to develop new formulations of fibre-rich functional foods.

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