PE9.41 Cattle Heart Hydrolysate Decreases Micellar Solubility and Cholesterol Absorption in Rats and Caco-2 Cells 398.00

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***Abstract*—the mechanism for the hypocholesterolemic action of a cattle heart protein hydrolysate (HPH) is clarified. The micellar solubility of cholesterol *in vitro* was significantly lower in the presence of HPH than in the presence of casein. The suppression of cholesterol uptake by Caco-2 cells was significantly higher in the cholesterol micelles containing HPH than in the cholesterol micelles containing casein. The serum cholesterol concentrations and atherogenic index were significantly lower in the rats fed with HPH than in those fed with casein. The cholesterol absorption measured by [^{3}H]-cholesterol was significantly lower by HPH feeding than by casein feeding in rats *in vivo* accompanying the changes in fecal steroid excretion. Thus, the hypocholesterolemic action of HPH involved the inhibition of jejunal cholesterol absorption. The cattle heart protein hydrolysate ultra-filtrate (HPHU, MW < ca. 1,000 Da peptide fraction) derived from HPH imparted stronger hypocholesterolemic activity than HPH in rats.**

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***Index Terms*—cattle heart, cholesterol, Caco-2 cells, livestock by-product.**

I. INTRODUCTION

Dietary proteins have been shown to influence serum cholesterol levels in many studies.[1-4)] Plant proteins such as soybean protein generally seem to have stronger hypocholesterolemic effects than animal proteins such as milk casein.[1)] Most of the animal and human studies on the effects of dietary proteins on serum cholesterol concentrations, however, have been focused on the differences between soybean protein and milk casein. Only a few reports have dealt with the effects of animal protein hydrolysate on cholesterol metabolism.

This study investigated whether dietary supplementation with an animal protein hydrolysate, cattle heart protein hydrolysate (HPH), would affect cholesterol metabolism in rats. Lacking any clear picture of the mechanism underlying the hypocholesterolemic action of HPH, we also decided to investigate the mechanism for HPH-induced hypocholesterolemia. To this end, we conducted *in vivo* studies and *in vitro* assays on the micellar solubility of cholesterol and cholesterol absorption in Caco-2 cells. Hypothesizing that a certain peptide fraction derived from HPH might induce a hypocholesterolemic action, we also attempted to identify a novel hypocholesterolemic fraction derived from HPH, in the hope of finding promising new uses for livestock by-products.

II. MATERIALS AND METHODS

Preparation of the cattle heart protein hydrolysate (HPH).

Cattle heart was obtained from Itoham Foods Inc. After removing fat tissues with a knife, the heart tissue was minced, mixed with water (2 parts of water to 1 part of minced tissue), boiled for 30 min at 100°C, and filtered through a 20-mesh screen while still hot. The filtered residue was collected, mixed with a 2-fold portion of water (residue:water=1:2 (w/w)), and adjusted to a pH value of 8.5 with $Ca(OH)_2$. The cattle heart residue was then hydrolyzed by alcalase (alcalase 2.4L; Novo Nordisk Pharma, Bagsvaerd, Denmark) at pH 8.5 and 55°C for 3 h, adding alcalase at a ratio of 1% to protein by weight. After heating at 90°C for 15 min and filtering the digest through a 20-mesh screen, the filtrate was collected, filtered through no. 1 filter paper, sampled to determine the protein content, and mixed with an equal portion of dextrin (dextrin:filtrate=1:1) to prepare for the spray drying

phase of the experiments. The spray drying preparation is identified as HPH.

Preparation of the cattle heart protein hydrolysate ultra-filtrate (HPHU).

HPH was further subjected to ultra-filtration with a 1,000 Da (MW)-cut filter membrane (Toyo Rosi Kaisya, Tokyo, Japan). The filtrate was collected and is identified as HPHU. The molecular weight of the filtrate was lower than ca. 1,000 Da.

Chemical analyses.

The protein content was determined by the Kjeldahl method.[5] Lipids were extracted by the method of Folch *et al*.[6] and weighed. The moisture content was determined as the loss in weight after drying at 105°C for 24 h. The ash content was determined by the direct ignition method (550°C overnight). The sugar content was evaluated by the deduction method. Casein was generously supplied by the Central Research Institute of Meiji Dairy Products Co. Ltd. The chemical compositions of casein, HPH and HPHU are given in Table 1.

Table 1. Chemical Compositions of Casein, HPH and HPHU

Component	Casein	HPH	HPHU
		g/kg	
Protein	86.0	42.9	81.3
Lipids	0	0.5	0.4
Sugar	1.5	49.1	3.5
Moisture	11.0	5.0	7.1
Ash	1.5	2.5	7.7

Serum, liver, and fecal lipid analyses.

The serum, liver, and fecal lipids were determined by the methods described previously.[7]

Animals and diets.

Male Wistar rats (Japan SLC, Hamamatsu, Japan) were used for the animal studies. The rats were housed in individual cages in an environmentally controlled room maintained at a temperature of 22±2°C with a 12-h cycle of light (8:00-20:00) and dark, the rats having free access to food and water. The detailed procedures for the animal experiments are described in the subsequent sections.

Effects of casein and HPH on micellar solubility of cholesterol in vitro *(Experiment 1).*

The micellar solubility of cholesterol with casein or HPH *in vitro* was measured by the previously described method,[7] with some modifications. The [^{14}C]-labeled micellar solutions (1.0 ml) were prepared at the following concentrations and mixed by sonication (VP-5 ultrasonic homogenizer, Taitec, Tokyo, Japan): 0.74 kBq [4-^{14}C]-cholesterol (2.1 Gbq/mmol, NEN, Boston, USA), 0.1 mmol/l of cholesterol, 6.6 mmol/l of sodium taurocholate, 1 mmol/l of oleic acid, 0.6 mmol/l of phosphatidylcholine, 0.5 mmol/l of monoolein, and 15 mmol/l of sodium phosphate (pH 7.4). After incubating at 37°C for 24 h, casein (casein sodium) or HPH (5 g/l, respectively) was added to the micellar solution, rendered soluble by sonication, incubated at 37°C for 1 h, and centrifuged at 100,000×g for 60 min at 37°C. The supernatant was collected for the determination of [^{14}C]-cholesterol by a liquid scintillation counter.

Effects of casein and HPH on the cholesterol absorption by Caco-2 cells in vitro *(Experiment 2).*

Caco-2 cells were acquired from the American Type Culture Collection and maintained as previously described.[7, 8] Monolayers were grown in 48-well plastic dishes containing 0.5 ml of fetal bovine serum supplemented with DMEM. The [^{14}C]-labeled micellar cholesterol uptake by Caco-2 cells was measured as previously described.[7, 8]

The [^{14}C]-labeled micellar solutions (0.2 ml) contained casein (casein sodium) or HPH (5 g/l, respectively). The cellular protein was determined by a commercially available protein assay kit (Bio-Rad, Tokyo, Japan). The amount of cholesterol absorbed by the cells is expressed as pmol/mg of protein.

Effects of casein and HPH on cholesterol absorption by rats in vivo *(Experiment 3).*

After acclimatizing to a commercial nonpurified diet (MF, Oriental Yeast, Tokyo, Japan) for 3 d, 9-wk-old rats weighing 185-205 g were deprived of food for 48 h, without restricting their access to water. Next, the rats received a test solution *via* intragastric intubation using a polyethylene catheter. One hour later, they were anesthetized with diethyl ether and killed. Blood was collected by cardiac puncture for separation of the serum, and the liver and intestines were quickly excised. The liver was rinsed with ice-cold saline, and the luminal contents of the small intestine were removed by flushing with ice-cold saline. The test

solutions consisted of 1 mmol/l of monoolein (Sigma), 5 mmol/l of taurocholic acid (Sigma), 37 kBq [1,2-^{3}H]-cholesterol (1972.1 GBq/mmol, NEN) and casein or HPH (60 mg) in 1ml of a 15 mmol/l phosphate buffer (pH 7.4). All of the solutions were emulsified by sonication. The [^{3}H]-cholesterol incorporated into the serum, liver, and intestine was extracted with hexane, after saponification with KOH-ethanol, as described previously.[7] Aliquots of the organic extract were used for scintillation counting.

Effects of dietary casein and HPH on the cholesterol metabolism of rats in vivo *(Experiment 4).*

After acclimatizing to a commercial nonpurified diet for 3 d, 5-wk-old rats 90-110 g weighing were divided into two groups of 6 rats each based on body weight. Each group had free access to one of the test diets containing casein or HPH as the protein source for 7 d. The composition of the basal diet was identical to that used in our previous study [7] and conformed to the recommendations of the American Institute of Nutrition.[9] Table 2 shows details of the experimental diet. Adjustment to the amount of carbohydrate (1 part of sucrose and 2 parts of gelatinized cornstarch) was used to compensate for the difference in dietary level of HPH. After 24 h of food deprivation, the rats were anesthetized with diethyl ether and killed. Blood was collected by cardiac puncture and the liver was removed. Fecal collection (7-9 d) for the determination of fecal steroid was completed before the 24-h food deprivation and blood sampling. The serum, liver, and fecal lipids were determined.

Table 2. Compositions of the Experimental Diets

Ingredient	Diet group		
	Casein	HPH	HPHU
	g/kg		
Casein [1, 2]	232.56	174.42	174.42
HPH [1]	–	116.60	–
HPHU [2]	–	–	61.50
Lard	50.00	50.00	50.00
Corn oil	10.00	10.00	10.00
Mineral mixture [3]	35.00	35.00	35.00
Vitamin mixture [3]	10.00	10.00	10.00
Choline chloride	2.00	2.00	2.00
Sucrose	181.90	181.20	180.49
Cornstarch	363.79	362.41	360.97
Cellulose	50.00	50.00	50.00
Lactose	0	0.87	0.87
Cholesterol	5.00	5.00	5.00
Sodium cholate	2.50	2.50	2.50
Dextrin	57.25	0	57.25

[1] Experiment 4. [2] Experiment 5. [3] AIN-93G diet

Effects of dietary casein and HPHU on the cholesterol metabolism of rats in vivo *(Experiment 5).*

After acclimatizing to the commercial nonpurified MF diet for 2 d, 5-wk-old rats 90-110 g weighing were divided into two groups of 6 rats each based on body weight. Each group had free access to one of the test diets containing casein or HPHU as the protein source for 10 d. The composition of the basal diet was identical to that used in our previous study [7] and conformed to the recommendations of the American Institute of Nutrition.[9] Table 2 shows details of the experimental diet. Adjustment to the amount of carbohydrate was used to compensate for the difference in dietary level of HPHU. After 24 h of food deprivation, the rats were anesthetized with diethyl ether and killed. Blood was collected by cardiac puncture, the liver was removed, and the serum and liver lipids were determined.

Statistical analyses.

Each result is expressed as the mean and SEM. The statistical significance of differences was evaluated by Student's *t*-test.[10]

III. RESULTS AND DISCUSSION

Micellar solubility of cholesterol in vitro (Experiment 1)

Figure. 1 shows the micellar solubility of cholesterol in the presence of casein or HPH. The micellar solubility of cholesterol was significantly lower in the presence of HPH than in the presence of casein.

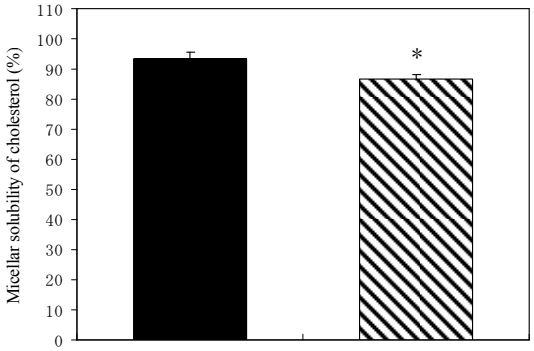

Fig. 1. Effects of Casein and HPH on the Micellar Solubility of Cholesterol *in vitro* (Experiment 1). Statistical significance compared to casein by Student's t-test (*P<0.05)

Cholesterol absorption in Caco-2 cells in vitro (Experiment 2)

Table 3. Effe
Distribution of

Serum[2]
Liver
Intestine

[1] Each value is
[2] Measured in a

Figure. 2 shows the cholesterol uptake from micelles containing casein or HPH. The cholesterol uptake from the micelles with HPH was significantly lower than that from the micelles containing casein.

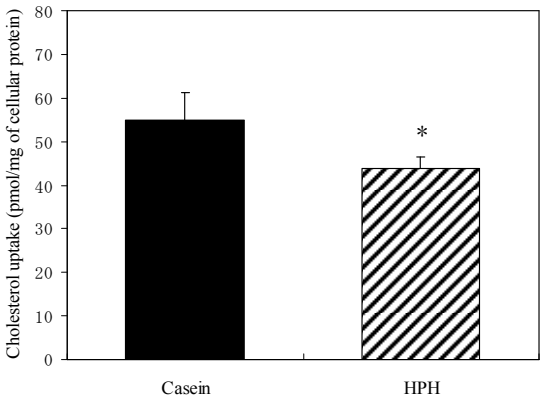

Fig. 2. Effects of Casein and HPH on the Cholesterol Absorption in Caco-2 Cells *in vitro* (Experiment 2). Statistical significance compared to casein by Student's t-test (*P<0.05)

Cholesterol absorption by rats infused with casein or HPH in vivo (Experiment 3)

The incorporation of [^{3}H]-cholesterol into the serum and liver was significantly lower in the HPH group than in the casein group (Table 3). The incorporation of [^{3}H]-cholesterol into the intestine tended to be lower（P=0.24）in the HPH group than in the casein group.

Effects of dietary casein and HPH on the cholesterol metabolism of rats in vivo (Experiment 4)

The body weight gain and food intake were unaffected by the dietary treatment (Table 4). The relative liver weight, serum total cholesterol and atherogenic index were significantly lower in the HPH group than in the casein group. Liver total lipid was significantly lower in the HPH group than in the casein group. The fecal output of cholesterol and total steroids was significantly higher in the HPH group than in the casein group.

Table 4. Effects of Dietary Casein and HPH on the Body and Relative Liver Weights, Food Intake, Serum and Liver Lipids, and Fecal Steroid Excretion in Rats after 7d (Experiment 4)[1]

	Diet group		
	Casein	HPH	
Body weight gain (g/7 d)	15.40 ± 0.90	14.20 ± 0.90	
Liver weight (g/100 g of body weight)	4.12 ± 0.04	3.82 ± 0.06	***
Food intake (day 3, g/d)	13.70 ± 0.50	13.80 ± 0.60	
Serum (mmol/l)			
Total cholesterol (a)	2.94 ± 0.11	2.50 ± 0.04	**
HDL-cholesterol (b)	0.42 ± 0.02	0.44 ± 0.02	
LDL+VLDL cholesterol [2]	2.52 ± 0.10	2.06 ± 0.03	**
Atherogenic index (a/b)	6.93 ± 0.26	5.70 ± 0.16	**
Liver (μmol/g of liver)			

Effects of dietary casein and HPHU on the cholesterol metabolism of rats in vivo (Experiment 5)

The body weight gain, liver weight, and food intake were all unaffected by the dietary treatment (Table 5). The serum total cholesterol was significantly lower in the HPHU group than in the casein group. The liver total lipid and cholesterol concentrations were significantly lower in the HPHU group than in the casein group.

Table 5. Effects of Dietary Casein and HPHU on the Body and Relative Liver Weights, Food Intake, Serum and Liver Lipids in Rats after 10 d (Experiment 5) [1]

	Diet group		
	Casein	HPHU	
Body weight gain (g/10 d)	25.80 ± 1.50	25.10 ± 1.50	
Liver weight (g/100 g of body weight)	3.86 ± 0.05	3.84 ± 0.05	
Food intake (day 6, g/d)	12.60 ± 0.40	12.80 ± 0.50	
Serum (mmol/l)			
Total cholesterol (a)	4.92 ± 0.30	3.20 ± 0.24	**
HDL-cholesterol (b)	0.72 ± 0.05	0.63 ± 0.07	
LDL+VLDL cholesterol [2]	4.20 ± 0.29	2.57 ± 0.25	**
Atherogenic index (a)/(b)	6.98 ± 0.48	5.35 ± 0.64	
Liver (μmol/g of liver)			
Total lipid	220.10 ± 7.60	196.40 ± 4.40	*
Cholesterol	108.60 ± 2.70	92.00 ± 3.20	**

[1] Each value is the mean ± SEM, n=6. Asterisks indicate difference from casein * P <0.05; **P <0.01.

[2] Values were calculated as follows: LDL + VLDL cholesterol = Total cholesterol – HDL cholesterol.

The level of serum total cholesterol in both HPH and HPHU groups was significantly decreased (-15% and -35% respectively) compared with the casein group

(Tables 5 and 6). Thus, HPHU clearly demonstrated a higher hypocholesterolemic effect than HPH and casein. After preliminary rat experiments to evaluate some proteins and protein hydrolysates derived from livestock and livestock by-products, we had identified the hypocholesterolemic action of HPH.

Serum cholesterol was significantly lower in the rats fed on a diet containing HPH than in those fed on a diet containing casein. Some have postulated, however, that the strength of the serum cholesterol-lowering activity depends on the degree of fecal steroid excretion (acidic steroids + neutral steroids). [11] The present study demonstrated a higher fecal excretion of total steroids by the rats fed on HPH, indicating that the effect was due, at least in part to an enhancement of fecal steroid excretion.

Cholesterol is rendered soluble in bile salt-mixed micelles and then absorbed.[12] In the present study, the micellar solubility of cholesterol was significantly lower in the presence of HPH than in the presence of casein. We were also very interested to discover the micellar solubility of cholesterol, a property reported to be significantly suppressed in the presence of soy protein hydrolysate,[7] β-lactoglobulin tryptic hydrolysate,[13] and egg ovomucin[14] *in vitro*. Sitosterol,[15] sesamin,[16] and catechin[17] also lowered the micellar solubility of cholesterol, in conjunction with the serum cholesterol-lowering effects in rats. When coupled with our results for HPH, these findings suggest that the suppressed micellar solubility of cholesterol inhibited cholesterol absorption in the jejunum. If this is so, the process may be closely tied to the action of these agents in lowering serum cholesterol. Several recent studies have used monolayers of a Caco-2 cell culture as a model system to examine the process of lipid metabolism.[18, 19] Our experimental system to evaluate cholesterol absorption with Caco-2 cells has proved to be very useful in clarifying the molecular mechanism underlying the inhibitory effect of HPH on cholesterol absorption from the small intestine, a mechanism hitherto unknown. In this present study, we found that HPH lowered the serum cholesterol concentrations in rats and inhibited the cholesterol absorption by Caco-2 cells as well as *in vivo* by using radiolabeled cholesterol. These results suggest that suppressed cholesterol absorption *via* the direct interaction between cholesterol-mixed micelles and HPH in the jejunal epithelia constitutes at least part of the mechanism for HPH-induced hypocholesterolemia.

We also attempted to identify a novel hypocholesterolemic fraction derived from HPH. HPHU (MW < ca. 1,000 Da peptide fraction) suggest that HPH retained its hypocholesterolemic activity even after it was further subjected to ultra-filtration. Importantly, HPHU derived from HPH exhibited a greater ability to decrease both the serum and liver cholesterol concentrations *in vivo* than HPH.

IV. CONCLUSION

This study has clearly identified a stronger hypocholesterolemic action in the presence of HPH and HPHU than in the presence of casein in our animal model. These findings on the hypocholesterolemic action of HPH and HPHU may lead to new uses for livestock by-products on an industrial level and for the promotion of health.

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