Plasma Cholesterol-Suppressing Effect of Papain-Hydrolyzed Pork Meat in Rats Fed Hypercholesterolemic Diet

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Summary The effects of papain-hydrolyzed pork meat on plasma and liver cholesterol levels were studied in rats fed a cholesterol-enriched diet. In rats fed the low-molecular-weight fraction of papain-hydrolyzed pork meat, the plasma cholesterol concentration, more particularly the VLDL and LDL cholesterol concentrations, were significantly lower (p<0.01) than in the rats fed untreated pork meat or soybean protein. Feeding with this fraction rather than with untreated pork meat also led to a significantly lower liver cholesterol concentration (p<0.01) and increased fecal excretion of neutral and acidic steroids. The low-molecular-weight fraction contained peptides with molecular weights of 3,000 or less and had an amino acid composition similar to that of pork meat itself. This study suggests that peptides produced by papain-hydrolysis of pork meat have a hypocholesterolemic activity through their interference with the steroid absorption process.

Key Words pork meat, protease digestion, plasma cholesterol, bile acid excretion, liver cholesterol

Dietary protein is one of the important factors determining the plasma cholesterol level and it is widely known that, in general, animal proteins produce a higher plasma cholesterol concentration than vegetable proteins. However, casein and soybean protein have been used in most conventional studies for comparison of animal with vegetables protein (1–3). There have been only a few investigations using pork meat protein (4–6). In recent years, the effects have been studied of protease-hydrolysis of peptides in dietary proteins on plasma cholesterol and it has been reported that the undigested peptides of soybean protein have a greater plasma hypocholesterolemic action than untreated soybean protein (7,8,10). Regarding

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animal protein, it has also been reported that a partial hydrolyzate of casein decreases the plasma cholesterol level more than intact casein does (9). However, Huff et al. showed that an enzymatic hydrolyzate of casein elevates serum cholesterol levels in the rabbit to much the same extent as intact casein (11); this effect however, was not pronounced.

In a previous paper, we reported that a papain-hydrolyzate of pork meat suppressed a raised cholesterol level in rats (12). In the present study, the effect of a soluble low-molecular-weight fraction of papain-hydrolyzated pork meat on plasma cholesterol was examined in rats.

MATERIALS AND METHODS

Preparation of papain-hydrolyzed pork meat. Pork meat protein was prepared by cutting swine dorsal musculus longissimus into pieces, defatting them with hexane and lyophilizing. This freeze-dried pork meat (PM) was used as an experimental source of protein for a control group. After adding water to adjust the protein concentration to 5%, PM was hydrolyzed using papain (Wako Pure Chemical Industries Ltd., Osaka, Japan) at pH 7.0 and 50°C for 24 h and then the papain was inactivated by heating to 90°C for 1 h (12). The papain-hydrolyzate thus obtained was passed through a gauze filter and centrifuged at 5,000×g for 20 min. The sediment was washed with water several times, and the insoluble fraction (ISF) obtained after freeze-drying. The filtrate was subjected to ultrafiltration through a membrane which separated molecular weights below and above 150,000 (Carbosep, Sumitomo Heavy Industries, Ltd., Tokyo). The permeable solution (outer) was freeze-dried as a low-molecular-weight fraction (LMF). The yields of ISF and LMF were respectively 50.3% and 46.1% (recovered dry weight percent).

Analysis of papain-hydrolyzed pork meat. PM, ISF and LMF were subjected to gel-filtration on a TSK-gel G-2000SW column (Toso Co., Tokyo) and the molecular weight of the constituents was determined. The chromatography patterns of PM, ISF and LMF are shown in Fig. 1. The diet was hydrolyzed with 6 N HCl under reduced pressure at 110°C for 24 h. After neutralizing, the amino acid composition was analyzed using HPLC (LC-6A, Shimadzu Co., Kyoto, Japan) (13) and the results are shown in Fig. 2.

Animals, diets and experimental design. The experiments were carried out on 5-week-old specific pathogen-free male Sprague-Dawley rats (Funabashi Farm Inc., Chiba, Japan). After the rats had been fed commercial pellets (type F2; Funabashi Farm) for 1 week, they were randomly allocated to one of four groups (5 rats each, each rat approximately 80 g body weight), each of which was to receive a different dietary protein. The composition of the basal diet was, in weight percent: protein 20, lard 5, mineral mixture 4, vitamin mixture 2, choline chloride 0.2, cellulose 5, cholesterol 1, sodium cholate 0.25 and cornstarch to 100. Vitamin and mineral mixtures according to Harper (14) were purchased from Oriental Yeast Co., Osaka, Japan. PM, ISF and LMF were used as the source of dietary protein and
Fig. 1. Gel filtration pattern on TSK-gel G2000 SW column of pork meat and its protease digestion products. MP and ISF were analyzed after being solubilized with SDS and eluted with 20% CH$_3$CN containing 0.1% TFA. Arrows indicate the elution volume of molecular weight markers. PM, untreated pork meat; ISF, insoluble fraction; LMF, low-molecular-weight fraction.

Fig. 2. Amino acid composition of pork meat and its protease digestion products.

added to the diet at the expense of cornstarch to produce a nitrogen level equivalent to that of soybean protein isolate (SPI; New Fujipro SE., Fuji Oil Co., Osaka, Japan). The rats were housed individually in an air-conditioned room (23–24°C, lights on 08:00 to 20:00). The rats were given the experimental diets for 21 days *ad libitum*. Food intake and body weight were recorded every day. Feces were collected for the last 2 days and lyophilized to enable analysis of neutral and acidic steroids. At the end of the experiment, after overnight (20:00–08:00 h) fasting, the rats were killed by withdrawing blood from the abdominal aorta under light diethyl ether anesthesia. The liver was excised immediately.

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Lipid analysis. The plasma total cholesterol, free cholesterol, phospholipid and triglyceride concentrations were analyzed by enzymatic assay using kits purchased from Wako Pure Chemical Industries. Liver lipids were extracted by the method of Folch et al. (15) and the cholesterol concentration was measured (16). Feces were saponified and neutral steroids and acidic steroids were analyzed by gas liquid chromatography (17, 18). Plasma lipoproteins were separated by ultracentrifugation (TL-100, Beckman Instruments Inc., CA, USA) as previously described (19) to obtain chylomicrons, as well as the very low-density lipoprotein (VLDL; $d < 1.006$), low-density lipoprotein (LDL; $1.006 < d < 1.063$) and high-density lipoprotein (HDL; $1.063 < d < 1.210$) fractions. The cholesterol concentration in each lipoprotein fraction was measured by an enzymatic method (20).

Statistical analysis. Values are given as mean and pooled SEM. Data were analyzed by one-way ANOVA followed by the inspection of all differences between pairs of means by Duncan’s new multiple range test when variances were homogeneous. Differences were regarded as significant at $p < 0.05$. When variances were heterogeneous (fecal coprostanol excretion), data were analyzed using a Kruskal-Wallis analysis and Wilcoxon test (21).

RESULTS

Table 1 shows body weight, food intake, food efficiency and liver weight of rats fed control (PM) or experimental diets. Body weight gain was significantly lower in rats fed the LMF than in rats fed PM, whereas the relative liver weight to body weight of the LMF group was significantly lower than in all other groups. Food intakes were the same for the various groups but the food efficiency of LMF was significantly lower than the PM.

Plasma and liver lipid concentrations and the cholesterol percentage of each lipoprotein fraction of the plasma are shown in Table 2. The LMF group showed

Table 1. Growth and liver weight of rats fed different sources of nitrogen for 21 days.

| Dietary groups | Body weight (g) | Food intake (g/day) | Food efficiency | Liver weight (g/100 g BW) |
|---------------|----------------|---------------------|-----------------|---------------------------|
|               | Initial | Gain  |                  |                   |                           |
| PM (5)        | 79     | 150$^a$ | 18.5              | 0.38$^a$         | 5.91$^{ab}$               |
| ISF (5)       | 80     | 145$^{ab}$ | 18.8              | 0.37$^{ab}$      | 6.25$^a$                 |
| LMF (5)       | 81     | 133$^b$ | 19.3              | 0.33$^b$         | 4.96$^b$                 |
| SPI (5)       | 80     | 137$^{ab}$ | 18.4              | 0.35$^{ab}$      | 5.51$^b$                 |
| Pooled SEM    | 3      | 4      | 1.2               | 0.021            | 0.14                      |

Values are the Mean and pooled SEM. $^{a,b,c}$ Values in the same column not sharing a common superscript letter are significantly different at $p < 0.05$. PM, intact pork meat; ISF, insoluble fraction of papain-hydrolyzed pork meat; LMF, low-molecular-weight fraction of papain-hydrolyzed pork meat; SPI, soybean protein isolate. Numbers of rats are in parentheses.
Table 2. Concentration of plasma and liver and percentage cholesterol content of each lipoprotein fraction from plasma of rats fed different sources of nitrogen for 21 days.

| Dietary groups | PM (5) | ISF (5) | LMF (5) | SPI (5) | Pooled SEM |
|---------------|--------|---------|---------|---------|------------|
| Plasma (mM)   |        |         |         |         |            |
| Total cholesterol |  7.16<sup>a</sup> |  6.10<sup>a</sup> |  3.03<sup>b</sup> |  5.90<sup>a</sup> |  0.38 |
| Free cholesterol |  0.81<sup>a</sup> |  0.70<sup>a</sup> |  0.40<sup>b</sup> |  0.73<sup>a</sup> |  0.05 |
| Triglyceride   |  3.32  |  3.41   |  2.99   |  2.63   |  0.28 |
| Phospholipid   |  2.68  |  2.78   |  2.86   |  3.13   |  0.19 |
| Percentage contents of cholesterol (%) |        |         |         |         |            |
| Chylomicron    |  43.9<sup>a</sup> |  43.3<sup>a</sup> |  37.6<sup>b</sup> |  43.5<sup>a</sup> |  1.07 |
| VLDL           |  28.0<sup>a</sup> |  23.1<sup>b</sup> |  13.2<sup>c</sup> |  28.4<sup>a</sup> |  0.62 |
| LDL            |  12.3<sup>a</sup> |  12.9<sup>a</sup> |  7.5<sup>e</sup>  |  9.1<sup>b</sup>  |  0.46 |
| HDL            |  13.8<sup>c</sup> |  18.0<sup>b</sup> |  39.4<sup>a</sup> |  16.0<sup>e</sup> |  0.75 |
| Liver (μmol/g) |        |         |         |         |            |
| Total cholesterol | 177<sup>a</sup> | 131<sup>b</sup> |  62<sup>c</sup>  |  122<sup>b</sup> |  4.17 |

Values are the means and pooled SEM. <sup>a,b,c</sup>Values in the same horizontal row not sharing a common superscript letter are significantly different at p<0.05. PM, intact pork meat; ISF, insoluble fraction of papain-hydrolyzed pork meat; LMF, low-molecular-weight fraction of papain-hydrolyzed pork meat; SPI, soybean protein isolate. Numbers of rats are in parentheses.

Table 3. Fecal excretion of neutral and acidic steroids of rats fed different sources of nitrogen.

| Dietary groups | Neutral steroids (mg/day) | Acidic steroids (mg/day) |
|---------------|---------------------------|--------------------------|
|               | Total     | Coprostanol<sup>a</sup> | Total     | Coprostanol<sup>a</sup> |
| PM (5)        |  76.6<sup>bc</sup> | 19.1±2.9<sup>b</sup>   |  25.1<sup>c</sup> |          |
| ISF (5)       |  71.4<sup>c</sup> | 52.6±5.3<sup>a</sup>   |  28.4<sup>bc</sup> |          |
| LMF (5)       |  100<sup>a</sup>  | 28.1±1.5<sup>b</sup>   |  90.5<sup>a</sup>  |          |
| SPI (5)       |  88.5<sup>ab</sup> | 23.0±1.3<sup>b</sup>   |  33.5<sup>b</sup>  |          |
| Pooled SEM    |  4.6       | 1.8                     |            |          |

Values are the means and pooled SEM. <sup>a,b,c</sup>Values in the same column not sharing a common superscript letter are significantly different at p<0.05. *Coprostanol was analyzed using a Kruskal-Wallis analysis and Wilcoxon test. Numbers of rats are in parentheses.

A hypocholesterolemic effect compared to the PM group. The cholesterol level of rats fed LMF was significantly lower even than those fed SPI, which is known to induce a lower cholesterol level than a casein diet. However, no such effect was observed on either the triglyceride or phospholipid levels. The concentration of liver cholesterol was also remarkably low in rats fed LMF. In rats fed SPI, the plasma cholesterol concentration was the same as that of rats fed PM, but less...
cholesterol accumulated in the liver. The cholesterol concentration of chylo-
microns, VLDL and LDL of rats fed LM was significantly lower than that of the
other groups. By contrast, the HDL fraction was not reduced, but in fact was
increased in the LMF group. These results indicate that the suppression of plasma
cholesterol concentration by LMF is due to its action on the chylomicron, VLDL
and LDL fractions.

Table 3 shows fecal steroid excretion of rats fed the control or experimental
diets. Fecal neutral steroid excretion was significantly higher in rats fed LMF than
in those fed PM and ISF. The ratio of coprostanol/total neutral steroid of rats fed
ISF was highest in compared to the other groups. There was a marked increase in
fecal acidic steroid excretion when the rats were fed LMF.

DISCUSSION

The present study showed that the LMF of papain-hydrolyzed pork meat has
a marked suppressing effect on plasma and liver cholesterol concentrations com-
pared to those seen with untreated PM. The chromatographic pattern (Fig. 1)
revealed that the major constituents of the LMF had molecular weights of approx-
imately 3,000 or less and they were represented by 9 peaks. A comparison with
standard specimens suggested that it contained peptides composed of several amino
acid residues. Our previous paper (12) discussed products artificially digested using
pepsin, trypsin and papain and reported that pepsin- and trypsin-hydrolyzed pork
meat had no plasma hypocholesterolemic effect. The low-molecular-weight fraction
of the pepsin- and trypsin-hydrolyzates contained larger peptides with many amino
acids (data not shown). This suggests that the plasma hypocholesterolemic effect
depends on peptides only found in papain-hydrolyzed PM.

Regarding the effect of dietary protein on plasma cholesterol concentration,
two main arguments have been discussed. One hypothesis is that the amino acid
composition of the protein, in particular the ratio of Lys/Arg (22, 23) and the
content of specific amino acids i.e. Met (24), Cys (25, 26) and Gly (27), affects
both the plasma amino acid composition and endocrine responses. However, the
amino-acid composition of LMF proved to be similar to that of PM and the
difference could not be explained in term of differences in Cys, Met and Gly content
alone (Fig. 2). The Lys/Arg ratio of the LMF was 1.24, close to that of PM,
(1.19). This suggests that the hypocholesterolemic effect of LMF was not the result
of a metabolic effect due to differences in amino acid composition.

The other hypothesis involves an intradigestive tract effect, i.e. the digestibility
of dietary proteins and the physicochemical properties of digestion products in the
digestive tract are related to cholesterol metabolism and are inherently different for
different proteins (28). For example, the hypocholesterolemic action of soybean
protein was found to be related to its low digestibility (29, 30). Thus, in this study,
the lower body weight gain of rats fed LMF may be an indication that LMF has a
low digestibility fraction that is not absorbed. Sugano et al. have reported that the

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insoluble high-molecular-weight fraction of the protease-hydrolyzate of soybean protein exerts a strong hypocholesterolemic activity through interference with the steroid absorption process (7, 8). It is suggested that the functional mechanism by which LMF suppresses the plasma cholesterol concentration is mainly related to intradigestive tract effects, since fecal excretion of neutral and acidic steroids was significantly higher. It is also possible that the indigestibility of LMF may increase the degradation of cholesterol. However, the measured alteration in the cholesterol content of the lipoprotein fractions may suggest that the LMF fraction influences cholesterol metabolism and thus the hypocholesterolemic effect of LMF depends also on mechanisms other than those involving the fecal excretion of steroids.

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