Effect of Partial Hydrolyzates of Casein and Soybean Protein on Serum Lipoproteins and Fecal Neutral Steroids

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Summary Effect of partial hydrolyzate of casein and soybean protein on serum lipoproteins and fecal neutral steroids in cholesterol-fed rats was studied. In rats fed partial hydrolyzate of casein, the levels of plasma and liver cholesterol, liver triglyceride and the ratio of serum \( \beta/\alpha \) lipoprotein had a tendency to decrease compared with those in rats fed intact protein. On the other hand, no difference was observed between soybean protein and partial hydrolyzate of soybean protein diet groups. The excretion of neutral steroids to feces and the amount of fecal coprostanol were increased in rats fed soybean protein and partial hydrolyzate of soybean protein.

Key Words casein, partial hydrolyzate of casein, soybean protein, partial hydrolyzate of soybean protein, cholesterol, lipoprotein

According to the development of the studies on lipoprotein metabolism, the role of apoprotein has been noticed. The effect of dietary protein on plasma cholesterol level has also been reported in the recent years (1–3). We observed that in the cholesterol-fed rats, high casein and soybean protein diet decreased plasma and liver cholesterol concentrations and increased high density lipoproteins (4). The difference of amino acid composition was demonstrated (5–8) but the presence of another unknown factor is considerable.

Casein is composed of three subunits and has 75,000 to 375,000 molecular weight. On the other hand, soybean protein is composed of several subunits and has 200,000 to 600,000 molecular weight (9). This examination was intended to investigate whether the structure and molecular size of the protein is responsible for the difference in lipoprotein metabolism.
METHODS

Male Sprague-Dawley rats weighing initially 100 g were controlled with commercial diet (type MF; Oriental Yeast Co., Ltd., Tokyo) for one week. At the start of the experiment, they were divided into four groups and were given experimental diets as shown in Table 1 for three weeks. Diets and water were provided *ad libitum*.

Table 1. Composition of diets. (%)

| Ingredient             | Percentage |
|------------------------|------------|
| Corn starch            | 65.175     |
| Lard                   | 5.0        |
| Protein*               | 20.0       |
| Vitamin mix. †         | 1.0        |
| Mineral mix. ‡         | 4.0        |
| Choline chloride       | 0.2        |
| Cellulose powder       | 4.0        |
| Sodium cholate         | 0.125      |
| Cholesterol            | 0.5        |

* Protein source was casein (C) (Oriental Yeast Co., Ltd., Tokyo), partial hydrolyzate of casein (PC), soybean protein (SB) (Ajinomoto Co., Ltd., Ajipron M2, Tokyo) and partial hydrolyzate of soybean protein (PSB). Each protein content was 85%. † Takeda Chemical Industries, Ltd., Panvitan powder. ‡ Oriental Yeast Co., Ltd., according to Harper.

![Fig. 1. Method of enzymatic hydrolysis of protein.](image-url)
Hydrolysis of casein and soybean protein was performed by the procedure described in Fig. 1. One hundred and fifty puk per g protein of Pronase E (Kaken Kagaku Co., Ltd., Tokyo) was added to 5% solution of each protein at 37°C and toluene was added in concentration of 0.1% to keep from rotting. Then casein and soybean protein solutions were incubated in a shaker for 2 and 20 h respectively. After the incubation each solution was heated at 85°C for 15 min to deactivate the
enzyme and then lyophilized. The protein concentration of both products was 85%. The percent of hydrolysis measured by the method of ultraviolet absorption (10) was about 60% (Fig. 2). The molecular weight of each hydrolyzate was less than 65,000 on SDS-polyacrylamide gel electrophoresis (11) as shown in Fig. 3.

Feces were collected for the last three days and dried to analyze the neutral steroids.

At the end of the breeding period, rats were fasted for 14–16 h. Blood was obtained by cardiac puncture to prepare serum and EDTA-plasma. Liver lipids were extracted by the method of Folch et al. (12). Plasma and liver cholesterol concentration was assayed by the method of Zak-Henly (13) and triglyceride concentration was measured by a kit (TG-test Wako, Wako Pure Chemical Industries, Ltd., Osaka). Polyacrylamide gel electrophoresis of serum lipoprotein was done by the method of Maruyama et al. (14). Neutral steroids were measured by the method of Miettinen et al. (15) using gas-liquid chromatography (model GC-6A, column model GC-9BM, Shimadzu Seisakusho, Kyoto).

RESULTS

The body weight gain, food intake, diet efficiency and liver weight are

| Table 2. Body weight, food intake, diet efficiency and liver weight. |
|---------------------------------------------------------------|
|                | Body weight (g)         | Food intake (g/day) | Diet efficiency | Liver weight (g) |
|                | Initial     | Gain   |               |               |               |
| C            | 5          | 139 ± 1* | 130 ± 4     | 19 ± 0.4     | 0.29 ± 0.01b | 13.1 ± 0.8b |
| PC          | 6          | 144 ± 3  | 131 ± 7     | 20 ± 0.9     | 0.28 ± 0.01b | 13.4 ± 0.6a |
| SB          | 7          | 142 ± 2  | 121 ± 6     | 20 ± 0.7     | 0.25 ± 0.01c | 9.7 ± 0.3c  |
| PSB         | 7          | 142 ± 2  | 128 ± 7     | 21 ± 0.5     | 0.25 ± 0.01c | 10.1 ± 0.4c |

* Mean ± SE. Values were significantly different: a > c, p < 0.01; b > c, p < 0.05.

| Table 3. Concentration of plasma and liver lipids. |
|--------------------------------------------------|
|                | Plasma                | Liver                |
|                |                       |                      |
|                | Cholesterol (mg/dl)   | Triglyceride (mg/dl) | Cholesterol (mg/g) | Triglyceride (mg/g) | Total lipid (%) |
| C            | 5  146.9 ± 23.0*     | 65.2 ± 13.8         | 36.7 ± 7.0       | 51.2 ± 16.0       | 10.9 ± 2.3       |
| PC          | 6  118.2 ± 7.6*      | 63.9 ± 11.4         | 30.1 ± 6.0       | 22.0 ± 6.3        | 8.9 ± 1.6        |
| SB          | 7  85.2 ± 3.4*       | 64.4 ± 8.4          | 43.4 ± 5.3       | 47.1 ± 7.3        | 13.3 ± 1.3       |
| PSB         | 7  90.5 ± 8.8*       | 84.8 ± 20.2         | 37.7 ± 7.0       | 50.0 ± 10.1       | 12.0 ± 2.5       |

* Mean ± SE. Values were significantly different: a > c, p < 0.01.

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presented in Table 2. Soybean protein (SB) and partial hydrolyzate of soybean protein (PSB) diet groups had lower diet efficiency and liver weight than casein (C) and partial hydrolyzate of casein (PC) diet groups.

Table 3 shows the concentration of plasma and liver lipids. The PC diet had tendency to have lower levels of plasma and liver cholesterol, liver triglyceride and the percentage of total lipid than the C diet. Plasma cholesterol concentration of rats given SB was lower than rats fed C and PC. There were no significant differences between SB and PSB diet group in the concentration of plasma and liver lipids.

Densitometric patterns of polyacrylamide gel disc electrophorograms of serum lipoprotein are shown in Fig. 4. At the top of the figures, the densitometric pattern of serum lipoprotein in a 110 g rat fed MF which have small preβ and β and large α fractions is shown. Instead of having clearly separated preβ and β fraction, all experimental groups had broad preβ and the β fraction had disappeared. The percentages of each fraction are shown in Table 4. PC diet group had a higher ratio of α₄ than the C diet group. The ratio of broad preβ over α fraction of C had tendency to show the higher level at 3.0 than that of PC, SB and PSB which had the

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Table 4. Polyacrylamide gel disc electrophorograms of serum lipoproteins.

|    | n   | pre\(\beta\) | \(\alpha_4\) | \(\alpha_3\) | \(\alpha_2\) | \(\alpha_1\) | pre\(\beta/\alpha\) |
|----|-----|--------------|--------------|--------------|--------------|--------------|----------------------|
| C  | 5   | 73.3±4.0\*  | 2.6±0.4\*   | 3.8±0.7      | 11.8±1.6     | 8.4±2.6      | 3.0±0.4              |
| PC | 6   | 65.6±3.3    | 6.1±0.7\b   | 7.6±1.3      | 16.3±1.4     | 7.0±1.4      | 2.0±0.3              |
| SB | 7   | 65.9±2.5    | 3.9±0.9      | 6.8±0.5      | 13.0±1.0     | 10.3±1.7     | 2.0±0.3              |
| PSB| 7   | 65.0±3.6    | 4.6±1.2      | 6.4±1.0      | 14.2±1.9     | 9.8±2.1      | 2.0±0.3              |

*Mean±SE. Values were significantly different: a<b, \(p<0.05\).

Table 5. Fecal excretion of neutral steroids.

| n   | Feces excreted (g/day) | Neutral steroids (mg/day) | Steroid excretion Cholesterol intake |
|-----|------------------------|---------------------------|-------------------------------------|
|     |                        | Coprostanol | Cholesterol | Total |                   |
| C   | 5                      | 3.49±0.78\* | 39.30±7.22\b | 42.78±7.89\a | 0.36±0.07       |
| PC  | 6                      | 3.96±0.39\b | 34.45±0.97\b| 38.41±0.75\d | 0.33±0.02\a     |
| SB  | 7                      | 36.07±5.21\c | 25.44±2.15\a| 61.51±7.22\c | 0.63±0.07\b     |
| PSB | 7                      | 38.48±2.59\a | 33.81±2.84 | 72.29±5.22\a | 0.57±0.04\c     |

*Mean±SE. Values were significantly different: a<b, \(p<0.05\); a<c, d<c, \(p<0.01\).

Gas-liquid chromatography analyses were performed as follows: column temperature from 170 to 230\degree C, carrier gas inlet pressure 1.8 kg/cm\(^2\), \(H_2\) flow rate 0.8 kg ml/min, air flow rate 1.0 kg liter/min, detector temperature 290\degree C.

same at 2.0.

Table 5 shows the excretion of neutral steroids to feces. The weights of feces in rats fed SB and PSB had tendency to a greater increase than in rats fed C and PC. Total neutral steroid excretion was higher in rats fed SB and PSB compared with rats fed C and PC. About 90\% of the neutral steroids was cholesterol in rats fed C and PC, while coprostanol occupied 54 and 52\% in rats fed SB and PSB respectively. The ratio of steroid excretion/cholesterol intake was higher in rats fed SB and PSB than rats fed PC.

DISCUSSION

The activity of Pronase E is the highest of all other protein digestive enzymes because of having non-specificity to the substrate. On the enzymatic hydrolysis of proteins with Pronase E in vitro, soybean protein takes ten times as long as casein (Fig. 1). This indicates that the digestibility of dietary soybean protein also seems to be lower than that of casein in vivo, though Pronase E is not a digestive enzyme of human or rat.

In our previous experiment using Wistar rats, the 40\% casein diet showed the
hypocholesterolemic effect. And on the pattern of polyacrylamide gel disc electrophoresis of serum lipoproteins, the percentages of \( \text{pre}\beta \) and \( \beta \) were smaller while those of \( \alpha_3 \) and \( \alpha_2 \) were higher in the 40% casein diet groups than in the 20% casein diet groups. It was also shown that the apoE, apoA-I and apoC percentages in HDL\(_2\) and apoE and apoA-I percentages in HDL\(_3\) had tendency to increase in rats fed the high casein diet (4). The high protein diet seemed to supply the amino acids or peptides which could be the source of apoproteins described above.

In this experiment using Sprague-Dawley rats, plasma cholesterol concentration and the ratio of serum \( \beta/\alpha \) lipoprotein had tendency to decrease in rats fed PC than rats fed C. Because of the prehydrolysis of C, free amino acids and peptides of PC would be more rapidly absorbed to synthesize the necessary apoproteins that could carry the absorbed cholesterol effectively than those of intact C. There is nonsignificant difference between C and SB in each plasma cholesterol level and the ratio of serum lipoprotein fraction in this experiment. It is because of the fact that sensitivity against lipids of the Sprague-Dawley rats is higher than that of the Wistar rats.

Free amino acids seemed to be absorbed at a higher rate than intact protein or partial hydrolyzate of protein. Huff \textit{et al.} (6, 8) reported that the amino acid mixture corresponding to casein gave similar plasma cholesterol level to the intact casein and the amino acid mixture corresponding to soybean protein gave higher plasma cholesterol level than the intact soybean protein on feeding the cholesterol-free diet in rabbits. Nagata \textit{et al.} (5, 7) also showed similar data in rats. In this experiment, PC had a tendency to show a lower level than casein, and PSB showed a similar cholesterol level to SB. The difference of diets which contains cholesterol or not should be considered, however, these data suggests that the form of protein in the intestine may affect the absorption and the cholesterol metabolism.

Fumagalli \textit{et al.} (16) concluded that the hypocholesterolemic effect of soya is probably due to its action in increasing the excretion of fecal neutral steroids. The excretion of neutral steroids to feces increased in rats fed SB and PSB than in rats fed C and PC (Table 3). Coprostanol increased about ten times as high in rats fed SB and PSB as in rats fed C and PC. However the ratio and the weight of coprostanol excretion were different from the results obtained by Nagata \textit{et al.} (5). These differences described above might have occurred by the difference of the diet whether it contains cholesterol or not.

Coprostanol is an unabsorbable sterol which is produced by intestinal bacteria (17) and the difference may be responsible for the change of intestinal flora between casein and soybean protein.

About the hypocholesterolemic factor other than the amino acid composition, further studies from multiple points of view are required.

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REFERENCES

1) Kritchevsky, D. (1979): Vegetable protein and atherosclerosis. *J. Am. Oil Chem. Soc.*, 56, 135–140.
2) Carroll, K. K. (1981): Soya protein and atherosclerosis. *J. Am. Oil Chem. Soc.*, 58, 416–419.
3) Okita, T., and Sugano, M. (1979): Effects of dietary proteins on plasma and liver lipids in rats. *Eiyo To Shokuryo (J. Jpn. Soc. Food Nutr.)*, 32, 397–401.
4) Tanaka, C., Sone, M., and Nozaki, Y. (1981): Effects of dietary protein on plasma lipoproteins and liver lipids in cholesterol-fed rats. *Eiyo To Shokuryo (J. Jpn. Soc. Food Nutr.)*, 34, 555–563.
5) Nagata, Y., Tanaka, K., and Sugano, M. (1981): Further studies on the hypocholesterolaemic effect of soya-bean protein in rats. *Br. J. Nutr.*, 45, 233–241.
6) Huff, M. W., and Carroll, K. K. (1980): Effects of dietary proteins and amino acid mixtures on plasma cholesterol levels in rabbits. *J. Nutr.*, 110, 1676–1685.
7) Nagata, Y., Tanaka, K., and Sugano, M. (1981): Serum and liver cholesterol levels of rats and mice fed soy-bean protein or casein. *J. Nutr. Sci. Vitaminol.*, 27, 583–593.
8) Huff, M. W., Hamilton, R. M. G., and Carroll, K. K. (1977): Effects of dietary proteins and amino acids on the plasma cholesterol concentrations of rabbits fed cholesterol-free diets. *Atherosclerosis*, 82, 275–277.
9) Smith, A. K., and Circle, S. J. (1972): Soybeans: Chemistry and Technology, The Avi Publishing Co., Inc.
10) Iwanaga, S. (1796): Tanpakushitsu no nodo sokutei, in Tanpakushitsu no Kagaku, I (in Japanese), ed. by Nihon Seikagaku Kai, Tokyo Kagaku Dojin Co., Ltd., Tokyo, pp. 53–55.
11) Weber, K., and Osborn, M. (1969): The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.*, 244, 4406–4412.
12) Folch, J., Lees, M., and Stanley, G. H. S. (1957): A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.*, 226, 497–509.
13) Zak, B., Dickenman, R. C., White, E. G., Burnett, H., and Cherney, P. J. (1954): Rapid estimation of free and total cholesterol. *Am. J. Clin. Pathol.*, 24, 1307–1315.
14) Maruyama, M., and Kobori, Y. (1972): Investigation of disc electrophoresis of serum lipoprotein. *Seibutsu Butsuri Kagaku* (in Japanese), 16, 95–97.
15) Miettinen, T. A., Ahrens, E. H., and Grundy, S. M. (1965): Quantitative isolation and gas-liquid chromatographic analysis of total dietary and fecal neutral steroids. *J. Lipid Res.*, 6, 411–424.
16) Fumagalli, R., Paoletti, R., and Howard, A. N. (1978): Hypocholesterolaemic effect of soya. *Life Sci.*, 22, 947–952.
17) Breusch, F. L. (1938): The fate of the plant sterols in the intestinal tract. *J. Biol. Chem.*, 124, 151–158.