Steroid-Binding Peptides from Dietary Proteins

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Summary The undigested high-molecular weight fraction (HMF) of soybean protein prepared after exhaustive digestion either by microbial proteases or by pepsin exerted a remarkable hypocholesterolemic activity compared to the parent protein in rats fed cholesterol-enriched diets. HMF bound in vitro with bile salts and stimulated fecal excretion of both neutral and acidic steroids far more than did soybean protein. Extraction of HMF with methanol slightly decreased the activity, but the methanol soluble fraction was not regarded as a principle determinant. Further degradation of the methanol-extracted HMF by various proteases resulted in loss of activity. Bile acid binding capacity of HMF from other vegetable proteins was lower than that from soybean protein.

Key Words soybean protein, protease digestion, undigested peptides, serum and liver cholesterol

Materials and Methods

Materials Soybean protein isolate (Fujipro R, Fuji Oil Co.) was exhaustively hydrolyzed by microbial proteases (Protin FC and AC, Daiwa Kasei Co.) at pH 7.0 and 50°C for 5h (1) or by porcine pepsin (Sigma Chemical Co.) at pH 2.0 and 37°C for 24h. The digest was heated at 80°C for 30 min and centrifuged (after neutralization in case of pepsin digestion). The sediment was washed with water twice and then the undigested high-molecular weight fraction (HMF) was obtained after freeze-drying. HMF was extracted with methanol at room temperature. The residue (HMF-R) was dried in vacuo. HMF-R was further hydrolyzed by various proteases, Protin AC, Pronase E (Sigma Chemical Co.) and trypsin (bovine pancreas, Difco Labs.) at pH 9.0 and 50°C for 6h. After neutralization, the reaction mixture was heated at 80°C for 30 min and freeze-dried (HMF-RD). Lipids, sugar and saponin were analyzed as described previously (1). The chemical properties and HPLC patterns on a TSKgel G3000SW column (Toso Co.) (1) of these preparations are shown in Table I and Fig. 1, respectively.

Animals and diets Young male Sprague-Dawley rats (Seiwa Experimental Animals) were used. The composition of the basal diet was by weight % (5): soybean protein 20, high-oleic safflower oil 10, mineral mixture 3.5, vitamin mixture 1.0, choline bitartrate 0.2, dl-methionine 0.3, cellulose 5, corn starch 15, cholesterol 0.5, Na-cholate 0.125, and sucrose to 100. When various protein digests were examined, they replaced soybean protein at an equivalent nitrogen level at the expense of sucrose. Rats were decapitated, blood was collected and the liver was excised.
Table 1. Chemical compositions of protease digestion products of soybean protein.

| Samples           | Moisture | Protein | Ash | Sugar | Lipid | Saponin |
|-------------------|----------|---------|-----|-------|-------|---------|
| Soybean protein   | 6.0      | 86.0    | 4.5 | 4.0   | 1.5   | 0.44    |
| Microbial protease digest |          |         |     |       |       |         |
| HMF               | 0.4-0.9  | 71.1-72.5 | 2.6-3.0 | 7.4-8.8 | 5.3-10.8 | 2.7-4.6 |
| HMF-E             | 1.2-2.7  | 34.1-36.2 | 5.6-6.6 | 7.1-8.3 | 27.7-33.9 | 8.6     |
| HMF-R             | 0.9-1.3  | 85.0-86.1 | 1.6-2.0 | 7.8-10.4 | 0.7-2.2 | 1.0     |
| HMF-RD            | 2.4      | 72.5    | 9.9 | 9.9   | 0.8   | 0.9     |
| Porcine pepsin digest |        |         |     |       |       |         |
| HMF               | 4.0      | 61.4    | 5.4 | 8.6   | 7.8   | 4.5     |

Values with the range of 2 or 3 preparations. 1 HMF: high-molecular weight fraction, HMF-E: methanol extract of HMF, HMF-R: residue of methanol extraction, HMF-RD: alkaline protease digest of HMF-R. 2 N X 6.25.

Table 2. Bile salts binding capacity in vitro of protease digestion products of soybean protein.

| Preparations   | Binding capacity (% bound) |
|----------------|---------------------------|
|                | Taurocholate   | Glycocholate |
| Cholestyramine | 92.5±0.2±6   | 86.8±0.0±6   |
| Soybean protein| 28.9±0.1±6b   | 19.0±0.3±6b  |
| Microbial HMF  | 36.1±0.2±6a   | 24.6±0.3±6a  |
| Microbial HMF-E| 29.9±0.3±6b   | 18.0±0.1±6b  |
| Microbial HMF-R| 29.0±0.7±6b   | 17.8±0.4±6b  |
| Microbial HMF-RD| 27.7±0.1±6b   | 17.9±0.3±6b  |
| Pepsin HMF      | 36.4±0.1±6c   | 23.6±0.4±6c  |

Values are mean±SE of 3 determinations. *-6 Non-matching superscripts in each column denote significant (p<0.05) difference.

were measured by gas-liquid chromatography (I).

Statistical analysis Data were analyzed by one-way analysis of variance followed by the inspection of all differences between pairs of means by Duncan's multiple-range test.

Results

Bile acid binding capacity in vitro Table 2 summarizes the bile acid binding capacity in vitro of various digestion products. The binding capacity was significantly higher in the 2 HMFs than in the other preparations. The binding capacity of HMF from various vegetable proteins was markedly lower than that from soybean protein (Table 3).

Effects of methanol treatment of HMF and HMF

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Table 3. Bile salt binding capacity in vitro of HMF from vegetable proteins.

| Protein sources | Binding capacity (µmol as taurocholate) |
|-----------------|----------------------------------------|
| Cholestryramine | 1.63                                   |
| Soybean         | 0.27                                   |
| Winged bean     | 0.18                                   |
| Wheat gluten    | 0.16                                   |
| Rice            | 0.15                                   |
| Potato          | 0.13                                   |
| Casein          | 0.10                                   |

Mean of duplicate determinations.

Table 4. Effects of various protease digests of soybean protein on serum and liver cholesterol.

| Experiments and nitrogen sources | Cholesterol |
|---------------------------------|-------------|
|                                 | Serum (mg/dl) | Liver (mg/g) |
| Soybean protein                 | 291 ± 11a    | 45.5 ± 2.2a  |
| Microbial protease digests      |             |             |
| HMF                             | 125 ± 5b     | 15.3 ± 1.4b  |
| HMF-E                           | 171 ± 12c    | 20.5 ± 1.5b  |
| HMF-R                           | 189 ± 17d    | 35.6 ± 5.2c  |
| Pepsin digests                  |             |             |
| M1H                             | 109 ± 5b     | 6.99 ± 0.70d |

Exp. 1

Exp. 2

Rats weighed on average 151 g (Exp. 1) and 165 g (Exp. 2) were fed experimental diet for 2 and 3 weeks. Values are mean ± SE of 6 rats. * Non-matching superscripts in each row denote significant (p < 0.05) difference.

Fig. 2. Fecal steroid excretion of rats fed various protease digests. Values are mean ± SE of 6 rats. a, b Non-matched letters in each column denote significant (p < 0.05) difference.

HMF-R.

Both types of HMF increased significantly fecal steroid excretion compared with soybean protein (Fig. 2). However, when HMF was treated with methanol, the stimulative effect disappeared both in HMF-E and HMF-R.

Effects of further digestion of HMF on serum and liver cholesterol Neither hypocholesterolemic nor liver cholesterol-lowering effect were observed in rats fed degraded HMF (HMF-R) compared to those fed intact soybean protein (Table 4, Exp. 2). HMF-RD by no means showed the stimulatory effect on fecal steroid excretion.

Discussion

The present study showed that HMF prepared after peptic digestion also effectively prevented the elevation of cholesterol in serum and liver by dietary cholesterol through interference with steroid absorption is also in the case of HMF prepared after microbial protease digestion (7). Thus, there is a possibility that these types of bile acid binding peptides are produced during gastric digestion.

The HMF preparations contained relatively large amounts of lipids (exclusively as phospholipids) and saponin. These contaminants are also

preparing after peptic digestion on serum and liver cholesterol As shown in Table 4 (Exp. 1), the elevation of serum and liver cholesterol by dietary cholesterol was markedly depressed when rats were fed both types of HMF. Because of the low nitrogen content (Table 1), HMF-E was diluted with soybean protein (70% of protein from soybean protein and 30% from HMF-E in the HMF-E diet). Both HMF-E and HMF-R were also effective in lowering serum and liver cholesterol, but to a lesser extent compared to intact HMF. The liver cholesterol-lowering effect of HMF-E was more efficient than
known to be hypocholesterolemic (7, 8). A major portion of these non-protein components was extracted with methanol and concentrated in HMF-E. This fraction was effective even though it was diluted with soybean protein. Alternately, the cholesterol-lowering activity of HMF-R was comparable to that of HMF-E. The serum and liver cholesterol lowering activity of soybean saponin at the level equivalent to that contained in the HMF diet was considerably lower than that of HMF, and no stimulating effect on fecal steroid excretion was observed in agreement with the reported data (9, 10). Thus, the effect of saponin is not primary but supplementary. In addition, the surprising hypocholesterolemic effect of HMF can not be attributed to the lipid component alone (8). Rather, it seems likely that the nitrogen components in HMF-E are responsible for its activity. When HMF-R was hydrolyzed by alkaline proteases, the hypocholesterolemic effect disappeared accompanying an increase in the proportion of the TCA soluble fraction (see Fig. 1) and a decrease in fecal steroid output. Thus, the peptides with relatively large molecular weights in HMF appear to have a role.

Judging from the HPLC pattern of various digests (Fig. 1), the peaks with molecular weight between $1 \times 10^3$ and $1 \times 10^4$ appear to be at least responsible for the hypocholesterolemic action of HMF.

The present study showed a positive relationship between the in vitro binding capacity and fecal steroid excretion, and hence, the hypocholesterolemic activity. However, HMF with bile acid binding capacity greater than that from soybean protein was not yet obtained from other vegetable proteins.

The effect of soybean protein on human cholesterol metabolism is rather variable and more moderate than in experimental animals (11, 12). Since the HMF exerts the significant hypocholesterolemic effect through the stimulation of fecal steroid excretion, attempts to use this type of preparation in humans are recommended.

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