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Differential digestibility of a synthetic slowly digestible peptide, oligo-L-methionine, in rats fed soybean protein or its hydrolysates

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Enzymatically synthesized oligo-L-methionine (OM), which consists of 5 to 12 polymerized L-methionine, is an insoluble, slowly digestible peptide. Digestibilities of diets with OM (3 g/kg diet) containing enzymatic hydrolysates of soybean protein isolate (SPI), large- and small-SPI peptides, were higher than those with OM added to an intact SPI diet at a low protein level (100 g/kg diet). The digestibility of OM was decreased when the content of these protein sources in diets was increased from a low level to a high level (200 g/kg diet). We examined the relation between the pancreatic protease secretion rate and the OM digestibility. Chymotrypsin secretion was strongly stimulated by feeding a low large-SPI peptide diet, followed by an SPI diet, and slightly enhanced by feeding a small-SPI peptide diet. The protease secretion was not correlated with OM digestibility. We also examined the inhibition of pancreatic digestion of OM by these protein sources. More than 20% of OM was digested in vitro by a low concentration of rat bile-pancreatic juice during a 1 hr incubation, which was evaluated by solubilization of a radiolabeled OM. The in vitro OM digestion was markedly inhibited by the addition of SPI and slightly inhibited by small-SPI peptides. The degree of inhibition by large-SPI peptide was between the values of SPI and small-SPI peptide. These protein sources inhibited the in vitro OM degradation in a dose-dependent manner. These results agree with in vivo OM digestibility. We conclude that the decreases in the inhibition of pancreatic digestion of OM improve OM digestibility more efficiently than the increase in the pancreatic protease secretion in rats fed SPI peptides. (J. Nutr. Biochem. 6:38–42, 1995.)

Keywords: peptide digestibility; oligo-L-methionine; pancreatic protease; rats

Enzymatically synthesized homopeptide of L-methionine, oligo-L-methionine (OM), consists of 5–12 polymerized L-methionine and the peptide is practically insoluble in water and almost organic solvents. The synthetic peptide is digested very slowly after ingestion in rats,[1] and is a useful probe for monitoring digestive function in the gut. We have examined the nutritional properties of the slowly digestible peptide and the effects of dietary factors on the digestibility of this peptide.

We previously observed that OM supplementation instead of free methionine to a low-casein diet prevents fatty liver induced by threonine imbalance.[2] Also, we observed that supplementation of OM to a low casein diet increased the growth rate of weaning rats, but the increase was absent or slight by supplementation of OM to a low soybean protein isolate (SPI) diet.[2] It has been demonstrated that the low supplementary effect of OM to a low SPI diet is due to low digestibility of OM in rats fed the SPI diet,[3] and also that the portal absorption of OM is lower after feeding a low SPI diet than after a low casein diet.[1,3] We provide evidence that the peptic digestion of OM in the stomach is partly involved in the differences in portal absorption of OM after feeding low casein and SPI diets, and pancreatic protease is also responsible for the OM digestion.[4] However, we showed that the pancreatic protease secretion after feeding a

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low SPI diet was not different from that after feeding a low casein diet.[5]

The purposes of the present study was to examine the effects of two enzymatic hydrolysates of SPI on the OM digestibility in rats, and to examine the effects of increasing the content of SPI and SPI hydrolysates in test diets on the OM digestibility. The enzymatic hydrolysates of SPI used in these experiments were large-SPI peptide, which was a peptic hydrolysate of SPI, and small-SPI peptide. We also examined the relationships between OM digestibility and increases in the pancreatic secretion rate after feeding, or inhibition in pancreatic digestion of OM by SPI or SPI peptides.

Methods and materials

Animals and diets

In vivo oligo-L-methionine digestibility. Male Sprague–Dawley rats (Japan SLC, Hamamatsu, Japan), weighing about 50 g, were fed a semipurified powdered diet[6–9] containing 250 g of casein/kg of diet (stock diet, shown in *Table 1*) for 3 or 4 days and divided into 6 groups of 6 rats by the randomized block design. In two sets of experiments, the rats were fed on low (100 g/kg diet) or high (200 g/kg diet) protein test diets, as shown in *Table 1*, for 14 days.

Table 1 Compositions of stock and test diets (g/kg diet)

		Low (or high) protein diets*		
	Stock diet	SPI diet	L-SPI diet	S-SPI diet
Casein†	250	—	—	—
SPI‡	—	100 (200)	—	—
Large-SPI peptide§	—	—	92 (192)	—
Small-SPI peptide§	—	—	—	101 (202)
Corn oil‖	50	50	50	50
Mineral mixture**	40	40	40	40
Vitamin mixture††	10	10	10	10
Granulared vitamin E‡‡	1	1	1	1
Choline bitartrate	4	4	4	4
Sucrose			to make 1 kg	

*Oligo-L-methionine (OM, 3 g/kg diet) was added to a low or high protein diet. Each low and high protein diet was isonitrogenous, respectively.
†Casein (ALACID; New Zealand Dairy Board, Wellington, New Zealand).
‡SPI (soybean protein isolate, Fujipro R, Fuji Oil Co. Ltd., Osaka, Japan).
§Sources of soybean protein isolate (SPI), large-SPI peptide (L-SPI) and small-SPI peptide (S-SPI) are described in Materials and methods.
‖Retinyl palmitate (7.66 μmol/kg diet) and ergocalciferol (0.0504 μmol/kg diet) were added to corn oil.
**The mineral mixture is prepared based on the AIN-76 Workshop held in 1989.[6] It provided (mg/kg diet): Ca, 4491; P, 2997; K, 3746; Mg, 375; Fe, 100; I, 0.32; Mn, 10.0; Zn, 34.7; Cu, 6.00; Na, 4279; Cl, 6542; Se, 1.05; Mo, 1.00; Cr, 0.50; B, 0.50; V, 0.25; Sn, 2.00; As, 1.00; Si, 20.0; Ni, 1.00; F, 2.72; and Co, 0.20.
††The vitamin mixture was prepared in accordance with the AIN-76 mixture[7] except that menadione[8] and L-ascorbic acid[9] were added to make 5.81 μmol/kg and 284 μmol/kg diet, respectively.
‡‡Vitamin E granule (Juvela, Eisai Co., Tokyo, Japan) supplied 423 μmol all-rac-α-tocopheryl acetate/kg diet.

These low and high protein diets contained SPI (Fujipro R, Fuji Oil Co. Ltd., Osaka, Japan), large-SPI peptide (peptic digest of SPI), or small-SPI peptide (Hynute PM, Fuji Oil Co. Ltd.) with or without OM (3 g/kg test diet). The content of nitrogen in both SPI-peptide diets were the same as the intact SPI diet. Feces were collected from days 12 to 14 after feeding test diets.

The distribution of molecular weight of large-SPI peptide and small-SPI peptide was estimated by gel permeation chromatography with high-performance liquid chromatography (HPLC) constructed with a Mini-Solvent Delivery System M 600 with Protein Pak 60 column ×2 (7.8 × 300 mm) (Waters, Milford, MA USA). The large-SPI peptide preparation contained more than 60% of a large peptide fraction (>1000 MW), and 74.5% of the small-SPI peptide preparation was fractionated to a small peptide fraction (<1000 MW). The amino acid composition of the two SPI peptides were similar to that of SPI (data not shown). The content of soybean trypsin inhibitor in SPI was negligible.

Oligo-L-methionine was prepared from L-methionine ethyl ester with papain as a catalyst according to a previously described method.[10,11] The synthesized OM was a mixture of 5 to 12 L-methionine peptides, which was estimated by the HPLC system described before with a C18 column (Wakosil II 5C18, 4.6 × 250 mm, Wako Pure Chemical Industries, Tokyo, Japan) to be methionine sulfone derivatives.[12]

Chymotrypsin secretion into bile-pancreatic juice after feeding test diets. Male Sprague–Dawley rats (Japan SLC), weighing about 250 g, were operated on to cannulate a common bile-pancreatic duct and to implant a duodenal cannula. These cannulas were connected behind the neck. Details of this procedure were described previously.[5] After 5 days of recovery period on a stock diet (*Table 1*), rats were fasted for 1 day and fed 2 g of a low protein test diet containing SPI, large-SPI peptide, or small-SPI peptide as shown in *Table 1*. Bile-pancreatic juice (BPJ) was collected for 3 min at 30 and 60 min before feeding and at 30, 60, 90, 120, 150, and 180 min after feeding under unrestrained condition. Bile-pancreatic juice was recirculated into the duodenum through the catheter continuously except during each 3 min sampling.

All the in vivo experiments were performed in a room controlled at 23 ± 2° C with a 12-hr light–dark cycle (800 to 2000 light period).

Analyses

Oligo-L-methionine excretion into feces (OM digestibility in vivo). Feces were freeze-dried and milled. Powdered feces were hydrolyzed in a mixed solution of HCl (6 mol/L) and formic acid (14.5 mol/L) at 110° C for 48 hr. Under these conditions, OM is hydrolyzed completely. The concentration of methionine in the hydrolysate was measured by an HPLC system as described before with a Wakopak WS-PTC column (4.0 × 200 mm; Wako Pure Chemical Industries) as phenyl thiocyanate (PTC) derivatives with phenyl isothiocyanate (Tokyo Kasei Kogyo, Tokyo).[13,14] We measured the amount of methionine in feces by one-step hydrolysis with a mixture of formic and hydrochloric acids. In the hydrolysate of OM with feces, about 95% of OM was recovered as methionine, and the fecal content of OM was corrected by the value of recovery. The OM added to the diet was also evaluated in the same manner.

Oligo-L-methionine excreted in feces was estimated by subtracting the average amount of methionine in feces of the groups fed the test diet without OM from that of individual rats fed a test diet with OM. Digestibility of OM (%) was calculated from the amounts of the ingested OM and the excreted OM in feces for 3 days.

Chymotrypsin activity in BPJ. The BPJ was diluted with 0.1% Triton X-100 solution adequately and activities of chymotrypsin was measured photometrically using a synthetic substrate N-benzoyl-L-tyrosine ethyl ester (BTEE).[15] Zymogens of chymotrypsin were activated by purified enterokinase (Sigma Chemical Co., St. Louis, MO USA).

In vitro degradation of OM by BPJ and inhibition by protein sources. In vitro OM degradation rate by BPJ was evaluated by solubilization of ^{14}C-labeled OM (718 kBq/mg OM). The radiolabeled OM was prepared using L-[methyl-^{14}C]L-methionine (American Radiochemical Inc., St. Louis, MO USA) by the method described above. The synthesized OM did not contain soluble peptides. Radiolabeled OM (1 or 2.5 g/L) was incubated in a Tris buffer (pH 8.0) at 37° C containing various concentration of BPJ and protein sources (*Figure 3*). The OM degradation rate was calculated by increases in soluble radioactivity. Soluble radioactivity obtained by an incubation without BPJ was very small, and we subtracted it from each value obtained by an incubation with BPJ. The solubilized OM by BPJ contained tetra or less chain length peptide of L-methionine (unpublished data). Intact OM was dissolved in 19 mol/L of formic acid and measured radioactivity. The radioactivity of the solubilized OM by BPJ and dissolved OM in formic acid were measured in a 15 mL scintillant consisting of p-terphenyl and p-bis(O-methylstyryl) benzene in toluene:ethyleneglycolmonoethyl ether (1:1) using a liquid scintillation system (Aloka, LSC 700, Tokyo, Japan).

Bile-pancreatic juice using the in vitro experiment was collected from rats fed the stock diet through a common bile-pancreatic catheter under pentobarbital anesthesia (40 mg/kg of body weight; Nembutal, Abbott, North Chicago, IL USA). The collected BPJ was activated by enterokinase (Sigma) at 30° C for 30 min and used in the in vitro OM degradation. Trypsin,[16] chymotrypsin, carboxypeptidase A,[17] and elastase[18] activities were estimated photometrically using synthetic substrates, Nα-p-toluenesulfonyl-L-arginine methyl ester (TAME), BTEE, carbobenzoxy-glycyl-L-phenylalanine (ZGP), and succinyl-trialanyl-p-nitroaniline (STANA), respectively.

Statistical analysis

The statistical analyses were performed by one-way and two-way (*Figure 1*, protein level and protein source) analysis of variance (ANOVA). The significant differences among means were determined by Duncan's multiple range test ($P < 0.05$).

Results

Table 2 shows body weight and food intake of rats fed low protein diets with or without OM. The growth rates of rats were not increased significantly by supplementation of OM in all the protein groups. The growth rates were not significantly different among the three OM supplemented groups. In the higher protein level, body weight gain and food intakes were not different among all the diet groups. Average values of body weight gain and food intake in rats fed high protein diets of all groups were 83.7 g/14 days ($n = 36$, $P = 0.5618$) and 230 g/14 days ($n = 36$, $P = 0.4054$), respectively.

Figure 1 shows OM digestibility in vivo in rats fed low or high protein diets containing OM. The OM digestibility of rats fed an SPI-based diet was about 30%, which was significantly lower than those of rats fed both large- and small-SPI peptide diets. The OM digestibilities of high protein groups were lower compared with those of corresponding low protein groups, respectively. The results of two-way ANOVA indicated that the effect of the protein level on the OM digestibility was significant ($P = 0.0022$). The digestibility of the high large-SPI peptide group was still higher than that of the high SPI group.

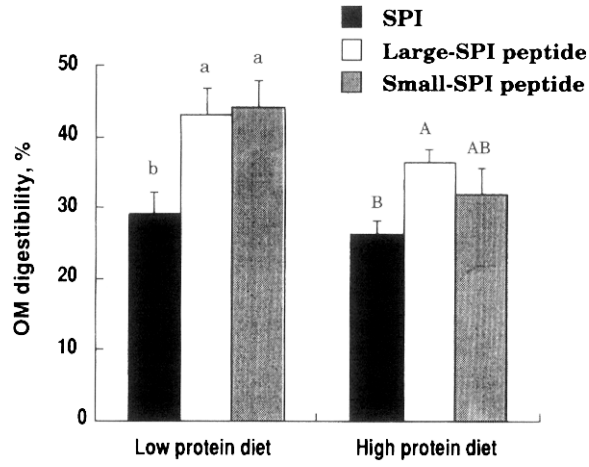


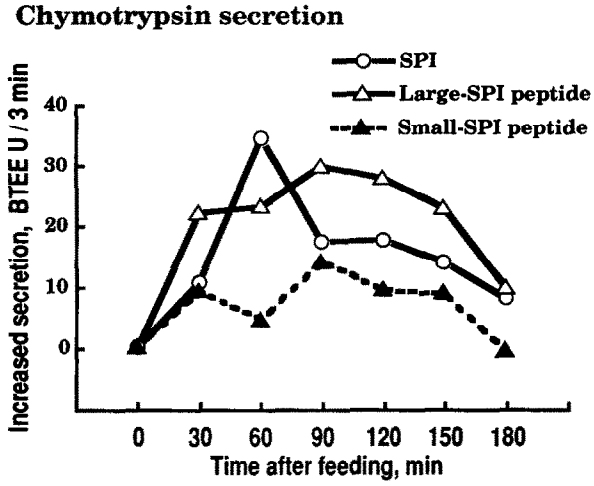
Figure 1 Oligo-L-methionine (OM) digestibilities in rats fed a low (100 g/kg diet) or high (200 g/kg diet) soybean protein isolate (SPI), large-SPI peptide and small-SPI peptide diets with or without OM (3 g/kg diet). The values were estimated from OM intakes and excretion into feces of OM for 3 days (days 12 to 14). Details are described in Materials and methods. All values are means with their standard errors for six rats. *P*-values estimated by two-way ANOVA were 0.0022 for protein level and 0.0003 for protein source. Mean values not sharing a common letter are significantly different between diet groups ($P < 0.05$).

Table 2 Body weight gain and food intake in rats fed low protein diets (100 g/kg diet) with or without oligo-L-methionine (OM; 3 g/kg diet)

Diet group	Body weight gain (g/14 days)	Food intake (g/14 days)
SPI	23.7 ± 4.5*	125.4 ± 10.1
Large-SPI peptide	38.5 ± 5.6†	154.9 ± 13.7
Small-SPI peptide	32.3 ± 4.3*†	141.1 ± 9.2
SPI + OM	34.3 ± 3.6*†	142.6 ± 9.0
Large-SPI peptide + OM	39.9 ± 4.5†	152.6 ± 9.2
Small-SPI peptide + OM	44.1 ± 1.8†	163.0 ± 5.0
ANOVA (*P*-value)	0.0328	0.1336

*†Means not sharing a common superscript within the same column are significantly different ($P < 0.05$, $n = 6$)

Figure 2 shows changes in chymotrypsin secretion in BPJ after feeding a low SPI, large-SPI peptide or small-SPI peptide diet. The increases in chymotrypsin secretion in the rats fed SPI or large-SPI peptide were higher than that in the rats fed small-SPI peptide. The profiles of the protease secretion was somewhat different among the three groups. So the integrated secretion for 180 min after feeding was evaluated based on the area under the curve of the upper panel of *Figure 2*. The value in the large-SPI peptide group was 3 fold higher than that in the small-SPI peptide group, as shown in the lower panel of *Figure 2*.



	Area under the curve (0-180 min) (U/3min)xmin
SPI	2920 ± 614 [ab]
Large-SPI peptide	3907 ± 548 [a]
Small-SPI peptide	1368 ± 725 [b]

Figure 2 Changes in chymotrypsin secretion in the bile-pancreatic juice above basal secretion after feeding of low soybean protein isolate (SPI), small-SPI peptide, or large-SPI peptide diet (100 g/kg diet) in conscious rats after 1 day fast. (Upper panel) Values are the amount of increased activity of the enzyme released from the pancreas for 3 min from the basal secretion. Values of basal secretion (secretion of the fasting state) are the average of two collections before feeding of a test diet. (Lower panel) The value of the area under the curve were calculated from the graph in the upper panel. *P*-value in ANOVA was 0.0440. Means not sharing a common superscript letter are significantly different ($P < 0.05$). All values are means with their standard errors for six rats.

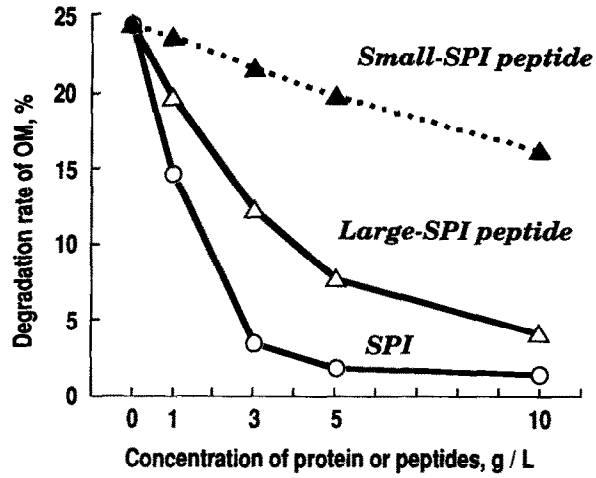


Figure 3 Effects of soybean protein isolate (SPI) or their enzymatic hydrolysates, large-SPI peptide and small-SPI peptide, on in vitro oligo-L-methionine (OM) degradation by diluted bile-pancreatic juice (10 g/L in medium). The assay was performed at 1 g/L of OM in Tris buffer (pH 8.0) at 37° C for 60 min. Values are means of three assays. Protease activities in the BPJ were as follows; trypsin, 201 TAME U/mL; chymotrypsin, 217 BTEE U/mL; carboxypeptidase A, 351 ZGP U/mL; and elastase 766 STANA mU/mL. One unit is defined as the activity of hydrolysis of the specific substrate at a rate of 1 μmol/min at 37° C.

The in vitro OM degradation (solubilization) rate was increased rapidly up to 10 g/L of BPJ in a medium, and then gradually increased thereafter in 1 and 2.5 g/L OM concentration. The percentage of degraded OM reached about 35% at 100 g/L of BPJ for a 1 hr incubation period. We confirmed that the degradation rate of OM was increased almost linearly for 60 min. The effects of SPI or SPI-peptides on the in vitro OM degradation were shown in *Figure 3*. The addition of SPI markedly reduced the degradation of OM by BPJ, but it was only slightly reduced by the addition of small-SPI peptide, and the degree of inhibition by large-SPI peptide was between those of SPI and small-SPI peptide. All the protein sources inhibit the in vitro OM degradation rate in a dose-dependent manner.

Discussion

The digestibility of a slowly digestible peptide, OM, in rats fed a diet containing a low level of intact SPI was only 30%, and the digestibility in the SPI group was clearly lower in the large- and small-SPI peptide groups (*Figure 1*). The result reveals that the low availability of OM in rats fed intact SPI is improved in rats fed enzymatically hydrolyzed SPI.

Figure 2 shows that the increase in chymotrypsin secretion of the SPI group was similar to that of the large-SPI peptide group. *Figure 2* also shows that the protease secretion in the large-SPI group was 3 fold higher than that of the small-SPI peptide group, even though OM digestibility in the large and small peptide groups was the same. Chymotrypsin is the most potent pancreatic protease in OM digestion (unpublished data), and we also show that other pancreatic enzyme secretion is parallel to chymotrypsin secretion after feeding a low protein diet.[4] These results reveal that the difference of the pancreatic protease secretion after feeding is not a major factor for determining in vivo OM digestibility.

From the results of ANOVA, the OM digestibility was higher in low protein groups than in high protein groups. In contrast, pancreatic protease secretion is known to be higher after feeding a high protein diet compared with that after a lower protein diet.[19] In our experimentally evaluated OM digestibility, the protease secretion in rats fed high protein diets may be higher than that in rats fed low protein diets, but the OM digestibility is lower in the high protein groups. These results also suggest that the pancreatic secretion rate does not determine the OM digestibility in vivo.

As shown in *Figure 3*, in vitro OM degradation by BPJ was inhibited by SPI or SPI-peptides in a dose-dependent manner. This result agrees with a decrease in the in vivo OM digestibility by the increasing protein content in diets. *Figure 3* also shows that in vitro OM degradation rates were higher in medium containing SPI peptides than in medium

containing intact SPI at the same concentration, which also agrees with the in vivo OM digestibility.

To inhibit the in vivo OM digestion, a considerable amount of SPI needs to be retained in the small intestine. Soybean protein is known to be digested and absorbed slowly,[1,20] which suggests that intact SPI or large fragments of SPI are retained in the small intestinal lumen for a long period of time after ingestion. This result suggests that ingested SPI inhibits OM digestion in the small intestinal lumen.

Figure 3 also shows that the inhibition of in vitro OM digestion by the small peptide was much less than that with the large peptide, even though in vivo OM digestibility in rats fed small-SPI peptide was similar to that in the large-SPI peptide groups. Chymotrypsin secretion after feeding a small-SPI peptide diet is markedly lower than that after feeding a large-SPI peptide diet. These results suggest that a similar OM digestibility between small- and large-SPI groups even though there is a lower inhibition by small-SPI peptide than by large-SPI peptide may be due to the lower protease secretion in the small-SPI peptide group. That is, in vivo OM digestibility is not only determined by the luminal inhibition of OM digestion by dietary protein.

In vivo digestibility of OM was less than 50%, which reveals that ingested OM reaches to the colon. So OM that reached the colon is possibly degraded by colonic bacteria, but the colonic degradation of OM may not contribute to the differences of OM digestibility among diet groups. The contribution of the colon to OM digestibility has to be evaluated.

We conclude that digestibility of a slowly digestible peptide, OM, in rats fed SPI is improved by hydrolysis of SPI to peptides, and that changes in luminal inhibition of pancreatic protease activity by dietary protein influenced more efficiently the digestibility of OM than those in the pancreatic protease secretion rate. Enzymatic hydrolysate may also improve the digestibility of other slower digestible proteins such as legume proteins.

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