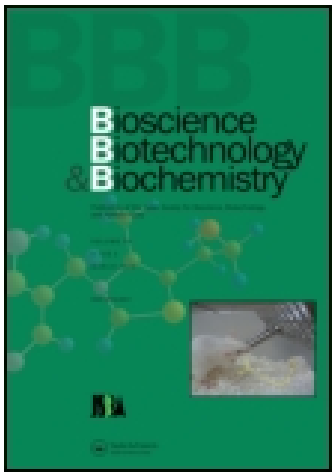

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Polymerization Degree of Oligomethionine to Determine Its Bioavailability When Added to a Low-protein Diets

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Polymerization Degree of Oligomethionine to Determine Its Bioavailability When Added to a Low-protein Diets

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Oligo-L-methionine ethylester (OMOEt) prepared by the papain-catalyzed oligomerization of L-methionine ethylester (MetOEt) is a mixture of pentamer to dodecamer and has nearly the same supplementary effect as free methionine (Met) for the growth of rats when added to a low casein diet, but its supplementary effect to a low-soy protein isolate (SPI) diet is not consistent and depends on the degree of polymerization. Rats were fed for 2 wk with an 8% casein or 10% SPI diet supplemented with 0.3% L-Met, each chemically synthesized Met_nOEt with a polymerization degree (n) of 6, 7, 8, or 9, or with OMOEt prepared by papain-catalyzed polymerization of MetOEt. Met_6OEt, Met_7OEt, and Met_8OEt had nearly the same supplementary effect on the growth of rats, as did free Met, both with the 8% casein and 10% SPI diets. The supplementary effect of Met_9OEt was not significantly lower than that of Met when added to the 8% casein diet, but was significantly lower when added to the 10% SPI diet. The digestibility of Met_9OEt supplemented to the 8% casein and 10% SPI diets was 50.5% and 35.6%, respectively. It appears likely that there is a gap in the bioavailability of oligomethionine between the octamer and nonamer when added to a low-protein diet, probably due to the rigidity of the structure increasing with the polymerization degree by α-helix formation. Although the differences in absorption rate of Met from OMOEt for a short time after feeding has been related to the different effects of supplemented OMOEt, the absorption rate of OMOEt for 30 min after feeding was not considered to be the main cause of the differential effects of OMOEt in this experiment.

Key words: oligomethionine; methionine; low-protein diet; liver triglyceride; rats

Oligo-L-methionine ethylester (OMOEt), prepared by the papain-catalyzed polymerization of L-methionine ethylester (MetOEt), is a mixture of oligomers with different degrees of polymerization[1-3] and shows interesting characteristics when added to low-protein diets given to rats. OMOEt has nearly the same supplementary effect as free L-methionine (Met) on the growth of rats when it is added to a low-casein diet, but has little supplementary effect on a low soy protein isolate (SPI) diet.[4] In a few cases, however, OMOEt has good supplementary effects on both low-casein and SPI diets. The inconsistent supplementary effect of OMOEt on a low SPI diet has been attributed to the difference in composition of OMOEt.[5] The polymerization degree of OMOEt had not been clearly determined (about 5[2] and 7.2–8.7 with 8 as major[3]), until we revealed by an HPLC analysis of OMOEt sulfone that OMOEt was a mixture of several oligomers with a polymerization degree of 5 to 12, with 7 or 8 as the major component, depending on the preparation.[5] We have demonstrated that OMOEt with a good supplementary effect on a low-SPI diet contained a higher ratio of the hexamer and heptamer compared to that with little supplementary effect on a low-SPI diet, and that there was a highly significant correlation between the molar ratio of oligomers with polymerization degrees of 5, 6, and 7 to total oligomers (polymerization degrees of 5 to 12) in OMOEt and the weight gain of rats fed on a low-SPI diet with OMOEt added.[5] This report describes the chemical synthesis of Met_nOET with polymerization degrees (n) of 6, 7, 8, and 9, and their supplementary effects on 8% casein and 10% SPI diets for the growth of young rats.

A low-casein diet (8–10%) supplemented with free Met (0.2%) causes liver fat accumulation and decreases in plasma threonine (Thr) and Serine (Ser) by Met-induced Thr imbalance.[6-9] The addition of OMOEt in place of Met to an 8% casein diet, however, did not produce fatty liver, although no significant difference was observed between the growth of rats fed on a low-casein diet supplemented with Met and OMOEt.[4] Therefore, the triglyceride content in the liver and plasma Thr and Ser are also described in this report.

Materials and Methods

Synthesis of Met_nOEt with polymerization degrees (n) of 6, 7, 8, and 9. The chemical syntheses of hexa-, hepta-, octa-, and nona-methionine ethylesters starting from Met are illustrated in the Figure. *N*-Boc-L-Met (Peptide Institute, Osaka, Japan) was used as a starting acid component for the synthesis of Met_nOEt ($n=6$, 7, 8, and 9), because the solubility of OMOEt in inorganic and organic solvents was very low, except in formic acid, and the Boc group could be removed by hydrochloric acid in formic acid. The carboxy group of the amine component was protected as an ethyl ester, because the carboxy terminal of OMOEt prepared by papain-catalyzed oligomerization of MetOEt was ethylesterified.

Coupling the acid and amine components was achieved by a mixed anhydride method with isobutyl chloroformate, because chloroethyl carbonate, which has been widely used for this purpose, sometimes gave urethane as a byproduct by a disproportionation reaction.[10]

Abbreviations: Boc-, *t*-butoxycarbonyl-; MetOEt, L-methionine ethylester; Met_nOEt, ethylester of oligo-L-methionine with polymerization degree n; OMOEt, oligo-L-methionine ethylester; SPI, soybean protein isolate. *Diet abbreviations*: C, 8% casein diet; S, 10% SPI diet; CM, 8% casein diet supplemented with 0.3% Met; SM, 10% SPI diet supplemented with 0.3% Met; COM, 8% casein diet supplemented with 0.3% OMOEt; SOM, 10% SPI diet supplemented with 0.3% OMOEt; CM_n, 8% casein diet supplemented with 0.3% Met_nOEt; SM_n, 10% SPI diet supplemented with 0.3% Met_nOEt.

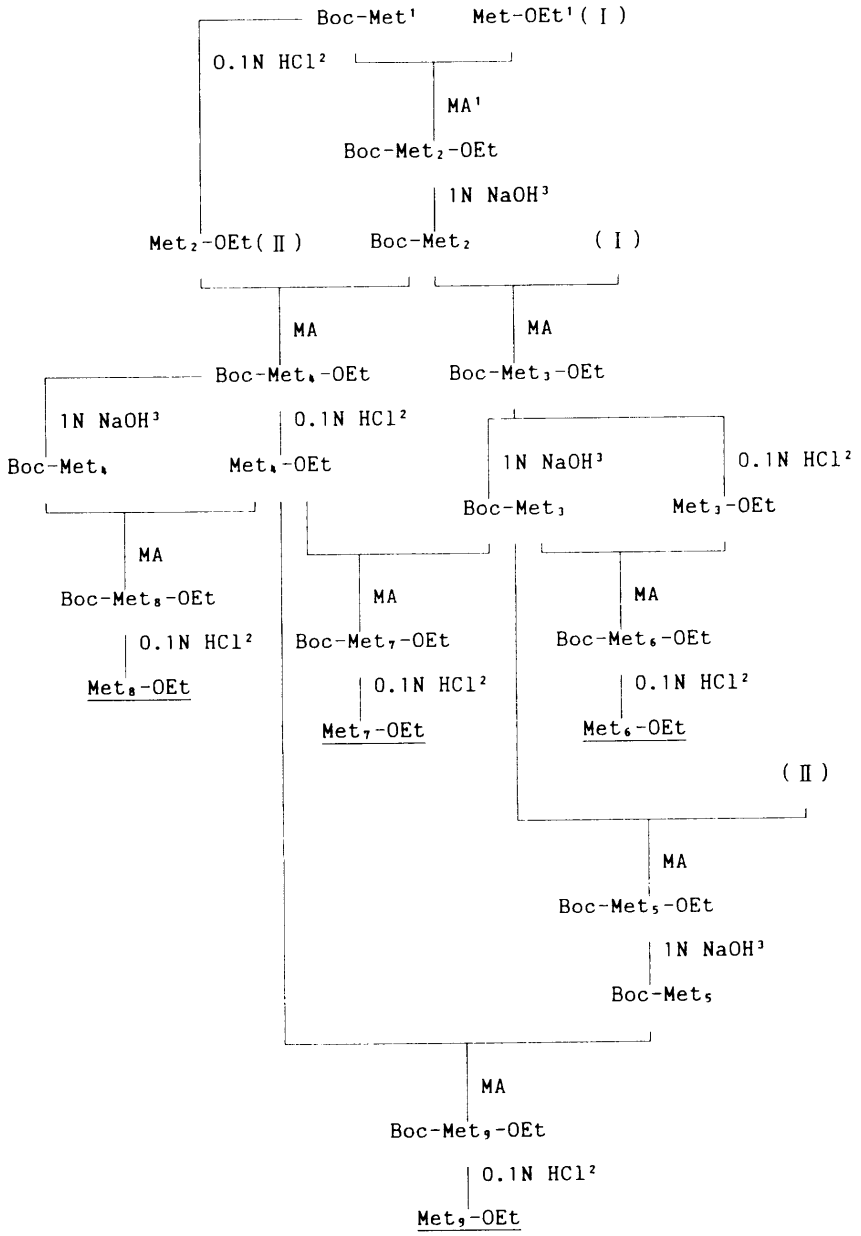

Fig. Synthetic Method for Producing Hexa-, Hepta-, Octa-, and Nona-L-methionine Ethylesters.

All methionine used was of L-form.
[1] BOC-Met, *t*-butoxycarbonyl-L-methionine; MetOEt, L-methionine ethylester; MA, mixed anhydride with isobutylchloroformate.
[2] In formic acid.
[3] In ethanol.

Sulfuric acid was used as an acid catalyst for the ethyl esterification of Met, because ethionine ethylester was formed as a byproduct when Met was esterified in the presence of hydrochloric acid.[11] Two hundred grams of Met was suspended in 1200 ml of anhydrous ethanol, and 146 ml of conc. sulfuric acid was added to the suspension. After refluxing for 3 h, the reaction mixture was allowed to stand overnight at room temperature and was neutralized by a solution of ammonium acetate in ethanol (212 g/about 800 ml). The precipitate formed was separated by filtration, and the filtrate was evaporated *in vacuo*. MetOEt sulfuric acid salt was obtained as the viscous residue from evaporation.

Met_nOEt ($n=6$, 7, 8, and 9) hydrochloric acid salts were synthesized as illustrated in Fig. 1, the preparation of Met_8OEt being briefly described as an example. Boc-Met_4 (12.8 g, 20 mmol), which had been prepared by coupling Boc-Met_2 and Met_2OEt and then by saponification with 1 N NaOH in ethanol, was dissolved in anhydrous dioxane (20 ml) and triethylamine (2.8 ml). Chloroisobutyl formate (2.6 ml) was added to the solution while stirring in an ice bath to form a mixed acid anhydride. Met_4OEt (12.3 g, 21 mmol), which had been prepared by coupling Boc-Met_2 and Met_2OEt and then by removing the Boc group with 0.1 N hydrochloric acid in formic acid, was suspended in anhydrous dioxane (300 ml) and triethylamine (2.8 ml), and then added to the mixed anhydride solution already prepared. The mixture was stirred vigorously and left overnight at room temperature. A white crystal of Boc-Met_8OEt was filtered off and washed with water. Yield: 14.3 g (52.5%); FDMS *m/z* (rel. int): 1217 ($M^+ + Na$, 52.5%), 1145 (1217 $-(CH_3)_3CO$ or $-COOEt + H$, 41), 1117 (1217 $-(CH_3)_3COCO + H$, 100). The Boc group was removed by an acid treatment, Boc-Met_8OEt (13.5 g, 11.3 mmol) being dissolved in 0.1 N hydrochloric acid in formic acid and allowed to stand for 1 h. After concentrating *in vacuo*, the residue from evaporation was suspended in water and centrifuged (3000 rpm). The washing procedure was repeated three times and the precipitate was lyophilized. Yield: 10.8 g (87.1%); FDMS *m/z* (rel. int): 1133 ($M^+ + K$, 15.4%), 1117 ($M^+ + Na$, 100), 1095 ($M^+ + H$, 17.6). Elemental analysis, FDMS and specific rotation data showed that the synthesized Met_nOEt samples ($n=6$, 7, 8, and 9) were pure and not racemic (Table I).

Feeding experiments were performed three times under essentially the same conditions, except that the experimental diets were given for 2 weeks in Experiments 1 and 2, and for 30 min in Experiment 3.

Table I. Elemental and FDMS Analyses of the Synthesized Oligomethionine Ethylesters and Specific Rotation of the Hydrolysate

	C	H	N	S	FDMS	$[\alpha]_D$ of the hydrolysate (6 N HCl)[1]
	(%)				(*m/z*, rel. int)	
Hexamer Found	43.15	7.19	9.28	21.46	869 (M^+ + K, 9.3), 856 (M^+ + Na, 100), 831 (M^+ + H, 44.2)	ND[2]
Calcd. for $C_{32}H_{61}N_6O_8S_6Cl$ ($Met_6OEt \cdot HCl \cdot H_2O$)	43.41	6.90	9.50	21.71		
Heptamer Found	43.98	6.99	9.52	22.13	1000 (M^+ + K, 9.3), 984 (M^+ + Na, 100), 962 (M^+ + H, 11.6)	+10.3
Calcd. for $C_{37}H_{68}N_7O_8S_7Cl$ ($Met_7OEt \cdot HCl$)	44.42	7.00	9.80	22.41		
Octamer Found	44.30	7.14	9.54	21.60	1131 (M^+ + K, 25.6), 1115 (M^+ + Na, 100), 1093 (M^+ + H, 11.6)	+12.5
Calcd. for $C_{42}H_{79}N_8O_{9.5}S_8Cl$ ($Met_8OEt \cdot HCl \cdot 1.5H_2O$)	43.54	7.08	9.68	22.12		
Nonamer Found	44.96	7.08	9.77	23.35	1262 (M^+ + K, 18.6), 1246 (M^+ + Na, 100), 1224 (M^+ + H, 7.0)	+12.7
Calcd. for $C_{47}H_{86}N_9O_{10}S_9Cl$ ($Met_9OEt \cdot HCl$)	44.71	6.98	9.99	22.83		

[1] $[\alpha]_D$ value for methionine hydrochloride is +12.7.
[2] Not determined.

Table II. Composition of the Experimental Diets for Experiments 1 and 2

	Diet[1]													
	C	CM	COM	CM_6	CM_7	CM_8	CM_9	S	SM	SOM	SM_6	SM_7	SM_8	SM_9
	(g/kg of diet)													
Casein[2]	80	80	80	80	80	80	80							
Soy protein isolate[3]								100	100	100	100	100	100	100
Methionine		3							3					
Met_6OEt (Experiment 1)				3							3			
Met_7OEt (Experiment 1)					3							3		
Met_8OEt (Experiment 2)						3							3	
Met_9OEt (Experiment 2)							3							3
OMOEt			3							3				
Mineral mixture[4]	40	40	40	40	40	40	40	40	40	40	40	40	40	40
Corn oil[5]	50	50	50	50	50	50	50	50	50	50	50	50	50	50
Choline bitartrate	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Vitamin E preparation[6]	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Vitamin mixture[7]	10	10	10	10	10	10	10	10	10	10	10	10	10	10
Sucrose	815	812	812	812	812	812	812	795	792	792	792	792	792	792

[1] C, 8% casein; CM, 8% casein supplemented with 0.3% Met; COM, 8% casein supplemented with 0.3% OMOEt; CM_n, 8% casein supplemented with 0.3% Met_nOEt; S, 10% SPI; SM, 10% SPI supplemented with 0.3% Met; SOM, 10% SPI supplemented with 0.3% OMOEt; SM_n, 10% SPI supplemented with 0.3% Met_nOEt.
[2] Alacid™, New Zealand Dairy Board, New Zealand.
[3] Fujipro R, Fuji Oil Co., Ltd., Osaka, Japan.
[4] The mineral mixture was identical to mineral mixture 2 (MM2) formulated by Ebihara *et al.*[19]
[5] Retinyl palmitate (7.66 μmol/kg diet) and ergocalciferol (0.0504 μmol/kg diet) were added to the corn oil.
[6] One gram of a vitamin E preparation (Juvelia; Eisai, Tokyo, Japan) provided 424 μmol of all-*rac*-α-tocopheryl acetate.
[7] The vitamin mixture was prepared according to the specification of the AIN-76 vitamin mixture (AIN 1977)[20] except for fat-soluble vitamins. Menadione and L-ascorbic acid were adjusted to 5.81 μmol/kg of diet (AIN 1980)[21] and 284 μmol/kg of diet (Harper 1959),[22] respectively.

Experiment 1.

Animals and diet. Male Sprague-Dawley rats (mean body weight of 50 g, Japan SLC, Inc., Shizuoka, Japan) were housed in individually suspended cages in a temperature-controlled room (23±1 C) with 12 h of light (0800–2000 h). They were fed with a 25% casein diet for 3 days before the experimental feeding period and were then divided into 10 groups of six rats each with a mean weight of 67 g. Each group was allowed free access to one of the following diets for 2 weeks: 8% casein diet (C), 10% SPI (Fujipro R, Fuji Oil Co., Ltd., Osaka, Japan) diet (S), 8% casein or 10% SPI diet supplemented with 0.3% Met (CM and SM, respectively), 8% casein or 10% SPI diet supplemented with 0.3% OMOEt prepared by papain-catalyzed oligomerization of MetOEt (COM and SOM, respectively), 8% casein or 10% SPI diet supplemented with 0.3% Met_6OEt (CM_6 and SM_6, respectively) and 8% casein or 10% SPI diet supplemented with 0.3% Met_7OEt (CM_7 and SM_7, respectively). The compositions of the experimental diets are summarized in Table II. Feces from the COM, CM_6, CM_7, SOM, SM_6, and SM_7 groups were collected for 2 days from days 12 to 14. Blood was obtained through the abdominal aorta under pentobarbital anesthesia (Nembutal, 5 mg/100 g body weight) on day 14. The rats were killed by exsanguination, before the liver was excised, weighed and stored frozen at −45°C until needed for analysis.

Undigested residues of methionine oligomers in the feces. The undigested residue of oligomethionine was measured to determine the digestibility of each oligomethionine. Since the amount of Met in the feces of the rats fed on the 8% casein diet was very small, the amount of Met in a hydrolyzate of the feces was assumed to be Met derived from undigested oligomethionine. The recovery of undigested Met_6OEt, Met_7OEt, and OMOEt from the feces as Met in the hydrolyzate of the feces, which was used as a factor for calculating the digestibility of each, was determined by hydrolyzing the feces of the 8% casein diet group supplemented with each of Met_6OEt, Met_7OEt, or OMOEt, respectively. The recovery (%) of Met_6OEt, Met_7OEt, and OMOEt determined as Met in the hydrolyzate of the feces was 76.4%, 74.4%, and 74.6% (mean of two measurements),

respectively. Since the solubility of OMOEt in 6 N hydrochloric acid was very low and the hydrolysis of OMOEt was incompleted, even after heating in 6 N hydrochloric acid for 48 h at 110 C in a sealed tube (unpublished data), and since OMOEt was soluble in formic acid, OMOEt was hydrolyzed in a mixture of conc. hydrochloric acid and formic acid (1 : 1, v/v). One milliliter of formic acid was added to lyophilized feces (10 mg). After being allowed to stand for 1 h, conc. hydrochloric acid (1 ml) was added, and the mixture was heated at 110 C for 48 h in a sealed tube. Met in the hydrolyzate was analyzed as the phenylthiocyanate derivative by HPLC with a Waters 600 multisolvent delivery system (Waters Associates) and Wako pak WS-PTC column (4.0 × 200 mm, Wako Pure Chemical Industries, Osaka, Japan).[12,13]

Liver triglycerides. Twenty milligrams of lyophilized liver was homogenized in a chloroform-methanol mixture (2 : 1, v/v; 1.7 ml) with a Potter homogenizer.[14] The filtrate was vigorously shaken with 0.37% KCl (0.4 ml) and left overnight, before the lower layer was concentrated *in vacuo*. The residue was dissolved in isopropanol (1 ml), and the triglyceride concentration was evaluated with a kit for measuring neutral lipid (TG-EN Kainos, Kainos Laboratories, Tokyo, Japan).

Plasma Thr and Ser. Plasma Thr and Ser were measured by the same method as that used for Met in the hydrolyzate of feces.

Experiment 2. Male Sprague-Dawley rats (mean body weight of 52 g) were fed on a 25% casein diet for 2 days and then divided into 10 groups of six rats each with a mean weight of 63 g. Each group was allowed free access to one of the following diets for 2 weeks: C, S, CM, SM, COM, SOM, 8% casein or 10% SPI diet supplemented with 0.3% Met_8 OEt (CM_8 or SM_8, respectively) and 8% casein or 10% SPI diet supplemented with 0.3% Met_9OEt (CM_9 or SM_9, respectively). Feces from the COM, CM_8, CM_9, SOM, SM_8, and SM_9 groups were collected for 2 days during days 12 to 14. Determination of the undigested residue of oligomethionine in the feces, blood collection from the abdominal aorta and the analysis of plasma Thr and Ser were done as in Experiment 1. The recovery (%) of Met_8OEt, Met_9OEt, and OMOEt determined as Met in the hydrolyzate of the feces, which was used for calculating the digestibility of each, was 67.9, 63.1, and 75.0 (means of two measurements), respectively.

Experiment 3. The rate of absorption of Met_nOEt and OMOEt for a short period (30 min) after feeding was examined according to the method of Chiji *et al.*[4] with a slight modification. Briefly, male Sprague-Dawley rats (mean body weight of 51 g) were fed on a 25% casein diet for 2 weeks, divided into 8 groups of six rats each with a mean weight of 133 g and then fasted for 24 h. Each group was fed 2 g of the 8% casein or 10% SPI diet with or without additional 3% Met_6OEt, Met_7OEt or OMOEt. Thirty minutes after feeding, blood was sampled from the portal vein and the abdominal aorta under anesthesia. The plasma Met concentration was measured, and the porto-aortic difference in Met concentration was calculated.

The study protocol was approved by the Hokkaido University Animal Use Committee, and the animals were maintained in accordance with the Hokkaido University-guidelines for the care and use of laboratory animals.

Statistical analyses. All data were subjected to ANOVA and tested by Duncan's multiple-range test to find whether the differences between means were significant ($p<0.05$). Values in the text are means ± SEM ($n=6$).

Results

Growth rate

No significant difference in weight gain was observed between the CM and COM groups, but the weight gain of the SOM group was significantly slower than that of the SM group in both Experiments 1 and 2 (Table III). Met_6OEt, Met_7OEt, and Met_8OEt had nearly the same supplementary effect as that of free Met for both the 8% casein and 10% SPI diets. The supplementary effect of Met_9OEt was not significantly lower than that of Met when

Table III. Body Weight Gain, Food Intake, Digestibility of Oligomethionine and Relative Liver Weight in Rats Fed with the 10% Casein or 10% SPI Diet Supplemented with 0.3% Oligomethionine[1]

Experiment 1	Diet[1]									
	C	CM	COM	CM_6	CM_7	S	SM	SOM	SM_6	SM_7
Body weight gain[3] (g/14 d)	15.7 ± 5.1^{c4}	39.5 ± 3.1^{ab}	31.0 ± 1.5^{b}	45.8 ± 5.0^{a}	48.9 ± 3.8^{a}	18.0 ± 2.4^{b}	49.8 ± 3.3^{a}	18.5 ± 3.4^{b}	50.3 ± 5.1^{a}	50.1 ± 3.3^{a}
Food intake (g/14 d)	111.6 ± 8.5^{c}	144.2 ± 7.7^{ab}	134.9 ± 4.5^{b}	157.3 ± 8.8^{a}	158.7 ± 8.0^{a}	114.8 ± 6.0^{b}	161.0 ± 4.6^{a}	119.1 ± 8.6^{b}	160.6 ± 9.2^{a}	162.0 ± 5.5^{a}
Digestibility of oligomethionine (%)			28.0 ± 2.0^{bC}	94.5 ± 0.7^{aA}	96.4 ± 0.5^{aA}			18.1 ± 4.7^{bD}	76.8 ± 1.3^{aB}	69.6 ± 2.3^{aB}
Liver weight (g/100 g of body weight)	5.25 ± 0.12^{bc}	5.70 ± 0.09^{ab}	5.13 ± 0.10^{c}	5.62 ± 0.24^{ab}	5.84 ± 0.19^{a}	4.73 ± 0.13^{b}	5.44 ± 0.12^{a}	4.97 ± 0.13^{b}	4.97 ± 0.11^{b}	4.83 ± 0.18^{b}

Experiment 2	Diet[2]									
	C	CM	COM	CM_8	CM_9	S	SM	SOM	SM_8	SM_9
Body weight gain[3] (g/14 d)	17.8 ± 2.7^{c4}	39.6 ± 4.4^{ab}	29.6 ± 2.4^{b}	44.4 ± 3.1^{a}	38.8 ± 1.2^{ab}	23.0 ± 2.1^{c}	58.5 ± 4.3^{a}	26.5 ± 3.7^{c}	59.4 ± 3.3^{a}	37.5 ± 2.3^{b}
Food intake (g/14 d)	111.3 ± 5.5^{c}	137.0 ± 9.1^{ab}	123.4 ± 7.7^{bc}	150.0 ± 5.9^{a}	141.8 ± 4.3^{a}	122.8 ± 6.9^{c}	167.4 ± 7.5^{a}	133.3 ± 6.5^{bc}	172.7 ± 5.9^{a}	148.6 ± 4.4^{b}
Digestibility of oligomethionine (%)			40.0 ± 4.4^{cD}	86.9 ± 2.3^{aA}	50.5 ± 2.6^{bC}			10.0 ± 3.7^{cE}	69.7 ± 2.8^{aB}	35.6 ± 3.6^{bD}
Liver weight (g/100 g of body weight)	5.21 ± 0.21	5.57 ± 0.14	5.05 ± 0.17	5.30 ± 0.13	5.23 ± 0.28	5.14 ± 0.19^{a}	5.24 ± 0.18^{a}	4.79 ± 0.12^{ab}	4.52 ± 0.07^{b}	4.56 ± 0.14^{b}

[1] Values are mean ± SEM ($n=6$).

[2] C, 8% casein; CM, 8% casein supplemented with 0.3% Met; COM, 8% casein supplemented with 0.3% OMOEt; CM_n, 8% casein supplemented with 0.3% Met_nOEt; S, 10% SPI; SM, 10% SPI supplemented with 0.3% Met; SOM, 10% SPI supplemented with 0.3% OMOEt; SM_n, 10% SPI supplemented with 0.3% Met_nOEt.

[3] The mean initial body weights were 67 g and 63 g for Experiments 1 and 2, respectively.

[4] Values in a row with a different superscript are significantly different by Duncan's multiple-range test ($p<0.05$). A superscript with a small letter indicates a difference in the casein- or SPI-based diets. A superscript with a capital letter indicates a difference in the combined casein- and SPI-based diet groups.

Table IV. Liver Triglycerides and Aortic Plasma Threonine and Serine in Rats Fed with the 8% Casein or 10% SPI Diet Supplemented with 0.3% Oligomethionine[1]

Experiment 1	Diet[1]									
	C	CM	COM	CM_6	CM_7	S	SM	SOM	SM_6	SM_7
Liver triglyceride (mg/g of liver)	41.78 ± 7.74[b3]	104.88 ± 5.19[a]	49.97 ± 4.93[b]	104.11 ± 10.85[a]	118.13 ± 5.25[a]	43.56 ± 8.80[bc]	87.36 ± 6.92[a]	36.63 ± 5.47[c]	67.80 ± 14.70[ab]	50.42 ± 9.89[bc]
Plasma threonine (μmol/liter)	339.6 ± 26.1[a]	65.8 ± 9.8[c]	141.3 ± 14.4[b]	63.6 ± 8.9[c]	46.5 ± 3.0[c]	482.3 ± 23.2[a]	45.0 ± 2.2[b]	454.5 ± 37.4[a]	44.4 ± 4.5[b]	52.6 ± 7.5[b]
Plasma serine (μmol/liter)	500.3 ± 20.1[a]	296.6 ± 13.0[b]	396.3 ± 26.0[a]	288.3 ± 27.4[b]	255.2 ± 11.7[b]	593.1 ± 44.3[a]	267.6 ± 17.8[b]	642.3 ± 33.9[a]	280.2 ± 16.0[b]	345.8 ± 19.1[b]

Experiment 1	Diet[2]									
	C	CM	COM	CM_8	CM_9	S	SM	SOM	SM_8	SM_9
Plasma threonine (μmol/liter)	355.5 ± 24.4[a]	46.5 ± 4.0[d]	118.6 ± 31.2[bc]	40.3 ± 5.7[d]	64.7 ± 15.3[cd]	567.4 ± 38.5[a]	38.6 ± 4.3[d]	463.3 ± 48.8[b]	88.0 ± 13.8[d]	244.6 ± 8.9[c]
Plasma serine (μmol/liter)	469.9 ± 15.2[a]	242.1 ± 7.5[cd]	353.1 ± 31.6[b]	230.9 ± 22.6[d]	307.4 ± 31.1[bc]	677.9 ± 49.0[a]	201.8 ± 10.2[d]	566.0 ± 33.4[b]	280.7 ± 16.8[d]	373.9 ± 13.2[c]

[1] Values are mean ± SEM ($n=6$).

[2] C, 8% casein; CM, 8% casein supplemented with 0.3% Met; COM, 8% casein supplemented with 0.3% OMOEt; CM_n, 8% casein supplemented with 0.3% Met_nOEt; S, 10% SPI; SM, 10% SPI supplemented with 0.3% Met; SOM, 10% SPI supplemented with 0.3% OMOEt; SM_n, 10% SPI supplemented with 0.3% Met_nOEt.

[3] Values in a row with a different superscript are significantly different by Duncan's multiple-range test ($p<0.05$).

added to the 8% casein diet, but was significantly lower than that of Met when added to 10% the SPI diet.

Digestibility of oligomethionine

The digestibility of OMOEt supplemented to the 10% SPI diet was lower than that supplemented to the 8% casein diet (Table III). Nearly all the Met_6OEt and Met_7OEt supplemented to the 8% casein diet was utilized by the rats. The digestibility of Met_6OEt and Met_7OEt supplemented to the 10% SPI diet was lower than when supplemented to the 8% casein diet, while the digestibility of Met_7OEt tended to be lower than that of Met_6OEt when added to the 10% SPI diet, although the difference was not significant. The digestibility of Met_8OEt added to the 8% casein diet was high, but the digestibility of Met_9OEt added to the 8% casein diet was significantly less when compared to that of Met_8OEt. The digestibility of Met_9OEt added to the 10% SPI diet was nearly half that of Met_8OEt.

Liver triglycerides

Triglycerides accumulated in the liver of the rats fed with the CM diet, but not in those fed with the COM diet (Table IV). Both Met_6OEt and Met_7OEt caused nearly the same accumulation of triglycerides in the liver as Met when added to the 8% casein diet. Liver triglycerides were less with increasing oligomerization degree when Met_nOEt was added to the 10% SPI diet. Liver triglycerides in the rats fed with the SM_7 diet were significantly lower than those fed with the SM diet.

Plasma Thr and Ser

Plasma Thr and Ser of the COM and SOM groups was significantly higher than the values for the CM and SM groups, respectively (Table IV). The plasma Thr level of the CM_6, CM_7, and CM_8 groups was decreased to the same level as that of the CM group, and that of the SM_6, SM_7, and SM_8 groups was also decreased to the level of the SM group. The plasma Thr level of the CM_9 group tended to be higher than that of CM_8, although the difference was not significant. Met_9OEt added to the 10% SPI diet, however, caused a significant increase in plasma Thr compared to the effect of Met_8OEt. Changes in plasma Ser followed those in plasma Thr.

In Experiment 3, the porto-aortic differences in Met concentration 30 min after feeding the casein- or SPI-based diets were as follows (μmol/liter): 5.22 ± 1.73^b, 16.25 ± 5.69^b, 39.10 ± 4.94^a, and 32.73 ± 4.51^a for the 8% casein diet, 8% casein diet supplemented with 3% OMOEt, 8% casein diet supplemented with 3% Met_6OEt, and 8% casein diet supplemented with 3% Met_7OEt, respectively, and 1.85 ± 3.56^b, 6.30 ± 0.97^b, 25.91 ± 5.01^a, and 10.03 ± 2.82^b for the 10% SPI diet, 10% SPI diet supplemented with 3% OMOEt, 10% SPI diet supplemented with 3% Met_6OEt, and 10% SPI diet supplemented with 3% Met_7OEt, respectively ($p<0.05$). The porto-aortic difference in Met concentration after feeding the casein diet with OMOEt tended to be higher than that in the rats fed with the SPI diet with OMOEt. The absorption of Met from Met_7OEt supplemented to the SPI diet was significantly lower than that from Met_6OEt with the SPI diet, but was no different from that from OMOEt with the SPI diet, whereas the absorption of Met from Met_7OEt and Met_6OEt was indistinguishable and significantly higher than that from OMOEt when added to the casein diet.

Discussion

OMOEt prepared by papain-catalyzed polymerization of MetOEt is a mixture of Met_nOEt with n of 5 to 12[5] and has nearly the same supplementary effect as that of Met for the growth of rats when added to a low-casein diet, although the supplementary effect with a low-SPI diet is inconsistent. In many cases, OMOEt has little supplementary effect with

a low-SPI diet,[4] and the difference in nutritional quality between casein- and SPI-based diets supplemented with OMOEt has been attributed to the different rates of luminal OMOEt digestion and Met absorption in the early stage of feeding.[4,15,16] Although several mechanisms has been proposed for the differences in absorption rate between OMOEt added to low-casein and low-SPI diets such as the response of exocrine pancreatic secretion and competition with dietary protein in the hydrolysis of OMOEt by proteases, the small intestinal transit is presumed to be important because it is faster after feeding a low-SPI diet than a low-casein diet, providing less time for the digestion and absorption of OMOEt fed with SPI than for that with casein.[17]

However, OMOEt sometimes has good supplementary effects with both the low-casein and -SPI diets, and OMOEt having a good supplementary effect with a low-SPI diet contains a higher ratio of oligomers with low polymerization degree (Met_5OEt to Met_7OEt) compared to that having little supplementary effect with a low-SPI diet.[5] It has been shown in this experiment that the different supplementary effect of OMOEt prepared by the papain-catalyzed oligomerization of MetOEt to low-casein and -SPI diets was caused by oligomers with a polymerization degree of 9 and above. Although the digestibility of Met_9OEt was also low in the CM_9 diet, Met absorbed from the CM_9 diet was enough to support the growth of the rats. The digestibility of Met_9OEt in the CM_9 and SM_9 diets was 50.5% and 35.6%, respectively (Table III). Therefore, the apparent amounts of Met supplemented to the 8% casein and 10% SPI diets were calculated to be 0.15% and 0.11%, respectively, which might explain the difference in effect between CM_9 and SM_9 on the growth of the rats. Since the weight gain of the rats fed with CM_9 tended to be lower than in those fed with the CM_8 diet, and since the digestibility of Met_9OEt in CM_9 was significantly lower than that of Met_8OEt in CM_8, it seems reasonable to assume that an oligomethionine with high polymerization degree would not support the growth of rats when added to a low-casein diet.

The growth of the rats fed with the COM diet was significantly faster than that of the control group, but tended to be slower than that of the rats fed with the CM diet in both Experiments 1 and 2, indicating that the OMOEt diet was utilized to some extent by the rats, but that the amount was not enough to support the growth of the rats when compared to those fed with the CM diet. OMOEt supplemented to the 10% SPI diet did not improve the growth of rats as reported previously.[4] From the digestibility of OMOEt in the COM and SOM diets, the apparent amounts of Met supplemented in the COM and SOM diet were calculated to be 0.08% and 0.05% in Experiment 1, and 0.12% and 0.03% in Experiment 2, respectively. Although the digestibility of OMOEt in the COM diet was higher in Experiment 2 than in Experiment 1, the growth of the rats in the COM diet group in Experiment 2 was slower than that in Experiment 1, while the digestibility of OMOEt in the SOM diet was higher in Experiment 1 than in 2, although the growth of the rats in the SOM group in Experiment 1 was slower than that in Experiment 2. The reason for these differences is not clear at this stage.

No liver triglyceride accumulation nor depression of plasma Thr and Ser caused by the low-casein diet supplemented with Met were observed in the rats fed on the low-casein diet supplemented with OMOEt, although the COM diet could support the growth of rats as reported previously.[4] Synthesized Met_6OEt amd Met_7OEt supplemented to the low-casein diet could not prevent the accumulation of liver triglycerides and the depression of plasma Thr and Ser, but plasma Thr and Ser of the CM_9 group were intermediate between the CM_8 and COM groups. A low-SPI diet supplemented with Met has also caused liver triglyceride accumulation.[4,9] The growth of rats fed with the 10% SPI diet supplemented with Met_6OEt and Met_7OEt was undistinguishable from those fed with the SM diet, but the liver triglycerides level of the SM_7 group was significantly lower than that of the SM group (Table IV). Slower absorption of Met from oligomethionine than that of free Met from the digestive tract may have been one of the reasons why the liver triglyceride accumulation was suppressed by supplementing with oligomethionine in place of Met to the low-protein diet.

The experimental results described here suggest a gap in the bioavailability of oligomethionine between Met_8OEt and Met_9OEt. Although the reason for this difference in bioavailability is obscure at this point, the increasing rigidity of the oligomethionine structure with higher polymerization degree by an α-helix formation may be one of the causes. The critical chain length for helix formation in oligomethionine is a heptamer.[18] Although the previous report described the differences in nutritional quality between a low-casein diet and SPI supplemented with oligomethionine, which was determined to be a mixture of Met_6OEt and Met_7OEt,[4] OMOEt used in these earlier experiments probably contained a fair amount of oligomethionine with a polymerization degree of 9 and above, judging from our results.

The higher absorption rate of Met from OMOEt supplemented to the casein diet than that from OMOEt added to the SPI diet during the 30-min period after feeding is related to the different effects of supplemented OMOEt in the casein- and SPI-based diets.[4] In Experiment 3, the absorption of Met from Met_7OEt added to the SPI diet 30 min after feeding was indistinguishable from that from OMOEt added to the SPI diet, and was significantly lower than that from Met_6OEt added to the SPI diet. The growth of rats fed with the SM_7 diet was, however, nearly the same as that by feeding with the fed SM_6 diet and was significantly faster than that by feeding the SOM diet. Therefore, the absorption rate of oligomethionine 30 min after feeding is not considered to be the main cause of the differential effects of supplemented OMOEt.

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