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# Dietary ε-Polylysine Decreased Serum and Liver Lipid Contents by Enhancing Fecal Lipid Excretion Irrespective of Increased Hepatic Fatty Acid Biosynthesis-Related Enzymes Activities in Rats.

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**ABSTRACT:** ε-Polylysine (EPL) is used as a natural preservative in food. However, few studies have been conducted to assess the beneficial functions of dietary EPL. The purpose of this study was to elucidate the mechanism underlying the inhibition of neutral and acidic sterol absorption and hepatic enzyme activity-related fatty acid biosynthesis following EPL intake. EPL digest prepared using an *in vitro* digestion model had lower lipase activity and micellar lipid solubility and higher bile acid binding capacity than casein digest. Male Wistar rats were fed an AIN-93G diet containing 1% (wt/wt) EPL or L-lysine. After 4 weeks of feeding these diets, the marked decrease in serum and liver triacylglycerol contents by the EPL diet was partly attributed to increased fecal fatty acid excretion. The activities of hepatic acetyl-coenzyme A carboxylase and glucose-6-phosphate dehydrogenase, which are key enzymes of fatty acid biosynthesis, were enhanced in rats fed EPL diet. The increased fatty acid biosynthesis activity due to dietary EPL may be prevented by the enhancement of fecal fatty acid excretion. The hypocholesterolemic effect of EPL was mediated by increased fecal neutral and acidic sterol excretions due to the EPL digest suppressing micellar lipid solubility and high bile acid binding capacity. These results show that dietary EPL has beneficial effects that could help prevent lifestyle-related diseases such as hyperlipidemia and atherosclerosis.

**Keywords:** ε-polylysine, lipid absorption, bile acid, fatty acid biosynthesis, rat

## INTRODUCTION

In western industrialized countries, recent lifestyle changes, including high-calorie diets and lack of physical exercise, have resulted in an increase in lifestyle-related diseases including atherosclerosis, hypertension, obesity, diabetes, and hyperlipidemia over the past few decades. The progression of lifestyle-related diseases is in part linked to lipid metabolism disorders and food intake patterns (1,2). Therefore, dietary therapy is important and should be considered as the first choice of treatment, or at least considered as important as medical treatment. Dietary proteins, such as plant and animal proteins, have been found to influence lipid metabolism in humans and animals (3-10). For example, soy protein and fish protein can improve hyperlipidemia through the enhancement of fecal lipid excretion and hepatic fatty acid oxidation activities (3-6,8). In addition, dietary basic proteins, such as ε-polylysine (EPL) and protamine, have been reported to decrease serum triacylglycerol (TG) levels through the inhibition of pancreatic lipase activity (11-15).

EPL is synthesized by linking the α-carboxyl groups of L-lysine with the ε-amino groups of another L-lysine molecule. EPL consists of approximately 25-30 L-lysine residues linked in this manner. Each amino acid residue in EPL contains a positively charged hydrophilic amino group. The production of EPL by natural fermentation is only observed in strains of the bacterial genus *Streptomyces* (16). EPL has been used as a natural preservative in food products for over 20 years in Japan and its safety has been confirmed (16-18). The use of EPL is common in foods such as boiled rice, cooked vegetables, soups, noodles, and sliced fish. EPL has been the subject of feeding studies of subchronic and chronic duration in rats using relatively high concentrations in the diet of

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50,000 and 20,000 ppm, respectively (19,20). In absorption, distribution, metabolism, and excretion studies on $^{14}C$-radiolabeled EPL, 92.9% of the dose of EPL passed unabsorbed through the gastrointestinal tract in to the feces, while some of the remainder was detected in the urine (1.2%) and expired air (3.0%) (17). In addition, excretion half-lives of EPL equivalents in blood and plasma were 20 and 3.9 days, respectively (17). Previous studies reported that EPL has an anti-obesity function in mice fed a high-fat diet by inhibiting the intestinal absorption of dietary fat (12,21). Therefore, EPL holds promise as a functional food ingredient to improve hypertriacylglycerolemia and obesity. However, few studies have focused on the influence of dietary EPL on serum and liver cholesterol contents and the enzyme activity related to hepatic fatty acid metabolism in experimental animal models. Possible findings about the effect of dietary EPL on cholesterol and fatty acid metabolism would be of great interest for the development of functional food ingredients. This study evaluated the effects of dietary EPL on serum, liver, and feces lipid parameters, and the activity of hepatic enzymes related to fatty acid metabolism. Moreover, to elucidate the mechanism underlying the inhibition of lipid absorption following EPL intake, we determined the physicochemical properties, including bile acid binding capacity, lipase activity, and micellar lipid solubility of casein and EPL digests prepared using a simulated gastrointestinal digestion model (3).

## MATERIALS AND METHODS

### Materials

EPL (EPL contained at 50.2% in dextrin, 25～30 lysine residues) was obtained from JNC Co. (Tokyo, Japan). L-lysine was obtained from Ajinomoto Co., Inc. (Tokyo, Japan). The ingredients for the experimental diets were purchased from Oriental Yeast Co. (Tokyo, Japan). All other chemicals and reagents were obtained from commercial sources and were of analytical grade.

### Preparation of casein and ε-polylysine digests by simulated gastrointestinal digestion

Casein and EPL were digested by the method of Hosomi et al. (3) with some modifications. Pepsin digestion parameters were as follows: protein concentration, 10% (wt/vol); enzyme/substrate ratio, 1:100 (wt/wt); and adjustment to pH 2.0 with HCl at 37°C. After incubation for 180 min, pepsin was inactivated by neutralization with $NaHCO_3$. Porcine pancreatin was added; pancreatin digestion parameters were as follows: enzyme/substrate ratio, 1:30 (wt/wt), with adjustment to pH 7.4 with $NaHCO_3$ at 37°C. For the monitoring of bile acid binding capacity, lipase activity, and micellar lipid solubility at intervals during digestion, aliquots of the digestion product were removed at 180, 182, 185, 190, 195, 200, 210, 225, 240, 300, and 360 min during the pancreatin *in vitro* digestion. Activities of aliquots of the digestion product were stopped by heating to 95°C for 15 min and then cooling to 4°C until analysis.

### Bile acid binding capacity

Bile acid binding capacities of the casein digest, EPL digest, and L-lysine were measured by the method of Takahashi et al. (11). The bile acid content was measured using an enzymatic assay kit (Total Bile Acid Test Kit; Wako Pure Chemical Industries Ltd., Osaka, Japan).

### Pancreatic lipase and cholesterol esterase activity

Pancreatic lipase and cholesterol esterase activities in the presence of casein digest, EPL digest, and L-lysine were determined by the method of Tsujita et al. (12) with some modifications. The lipase and esterase reaction mixtures (200 μL) contained 5 g/L digest products and L-lysine, and the concentrations of other components followed a previous study (12). After the lipase or esterase was added to the mixture, incubation was carried out at pH 7.0 and 37°C for 30 min. The amount of fatty acid produced was determined by an enzyme colorimetric method using the NEFA C-test Wako (Wako Pure Chemical Industries Ltd.). The lipase and esterase activities in the presence of casein digest, EPL digest, and L-lysine were defined as free fatty acid contents in the reaction solutions.

### Micellar lipid solubility

Micellar lipid solubility in the presence of casein digest, EPL digest, and L-lysine was measured by the method of Nagaoka et al. (4) with some modifications. The micellar solutions were incubated at 37°C for 24 h and then 0.5 mL of digest products and L-lysine (0.05 g/mL) were individually mixed with 0.5 mL of micellar solutions. Micellar solutions (0.5 mL) containing 6.6 mmol/L sodium taurocholate, 1.5 mmol/L cholesterol, 1 mmol/L oleic acid, 0.5 mmol/L monoolein, 0.6 mmol/L phosphatidylcholine, 132 mmol/L NaCl, and 15 mmol/L sodium phosphate (pH 7.4) were treated by sonication. The mixture was incubated at 37°C for 1 h, and then ultracentrifuged at 100,000 *g* for 1 h at 37°C. The supernatant was collected for the determination of cholesterol, fatty acid, phospholipid, and bile acid concentrations using an enzymatic assay kit (Cholesterol-E-Test Wako, Wako NEFA C-test kit, Phospholipids C-test Wako, and Total Bile Acid Test Kit; Wako Pure Chemical Industries Ltd.).

**Animal care and diet**

The experimental protocol was reviewed and approved by the Animal Ethics Committee of Kansai Medical University (Osaka, Japan) and followed the "Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions" (Notice No. 71, issued by the Japanese Ministry of Education, Culture, Sports, Science and Technology, June 1, 2006). Male Wistar rats (4 weeks old) were obtained from Japan SLC, Inc. (Shizuoka, Japan). The animals were kept in an air-conditioned room (temperature, 21～22°C; humidity, 55～65%; lights on, 08:00～20:00). Rats were acclimatized for 3 days with *ad libitum* access to tap water and control diet prepared according to the AIN-93G formula (22). After acclimation, rats were divided into 3 groups of 7 rats each with similar mean body weights and received the diets of control, EPL, and L-lysine-amino acid (LYS-AA). The composition of the experimental diets is shown in Table 1. The EPL diet contained 19.92 g/kg EPL, which included 50.2% EPL and 44.6% dextrin (wt/wt). The LYS-AA diet contained 10 g L-lysine/kg.

Food and water consumption and body weight were recorded every 2 days. Feces were collected from each group every 24 h for 7 days before sacrifice. After receiving the experimental diets for 4 weeks, the rats were weighed and sacrificed while anesthetized using pentobarbital (Nembutal®; Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan). Blood was collected from the abdominal descending aorta without anticoagulants, and serum was subsequently obtained by centrifugation of the blood at 2,500 *g* for 15 min. Liver, epididymal white adipose tissue (WAT), mesentery WAT, and perirenal WAT were rapidly removed, weighed, rinsed with cold saline, and then frozen in liquid nitrogen, followed by storage at −70°C until analysis.

**Table 1.** Composition of the experimental diets (unit: g/kg)

| Components | Dietary group | | |
|---|---|---|---|
| | Control | EPL | LYS-AA |
| Casein | 200 | 190.08 | 190 |
| ε-Polylysine[1)] | - | 19.92 | - |
| L-Lysine | - | - | 10 |
| L-Cystine | 3 | 3 | 3 |
| Dextrinized corn starch | 132 | 122 | 132 |
| Corn starch | 397.5 | 397.5 | 397.5 |
| Sucrose | 100 | 100 | 100 |
| Cellulose | 50 | 50 | 50 |
| Soybean oil | 70 | 70 | 70 |
| AIN-93G mineral mixture | 35 | 35 | 35 |
| AIN-93 vitamin mixture | 10 | 10 | 10 |
| Choline bitartrate | 2.5 | 2.5 | 2.5 |

[1)]ε-Polylysine contained 50.2% ε-polylysine and 44.6% dextrin (wt/wt).
EPL, ε-polylysine; LYS-AA, L-lysine-amino acid.

**Analysis of lipid composition**

Serum phospholipid, TG, cholesterol, high-density-lipoprotein (HDL) cholesterol, and non-HDL cholesterol were measured using an Olympus AU5431 automatic analyzer with AU reagent (Beckman Coulter Inc., Brea, CA, USA).

Total liver lipids were extracted according to the method of Bligh and Dyer (23). Each total lipid sample was dissolved in 2-propanol, and the TG content was determined using an enzymatic assay kit (Triglyceride E-Test Wako, Wako Pure Chemical Industries Ltd.). Liver cholesterol content was analyzed using a gas-liquid chromatography system (GC-14B, Shimadzu Co., Kyoto, Japan) equipped with a SE-30 column (Shinwa Chemical Industries, Kyoto, Japan) in which 5α-cholestane was used as an internal standard. Liver phospholipid content was measured by phosphorus analysis (24).

Feces were dried to a constant weight and then ground to a fine powder. Fecal fatty acid content was determined by the method of van de Kamer et al. (25). The level of fecal neutral sterol, including cholesterol and coprostanol, was determined by gas-liquid chromatography as described above. Fecal acidic sterol content was determined as micromoles of 3α-hydroxysteroid based on the molar extinction coefficient of NADH at 340 nm (26). Fecal nitrogen content was determined by the Kjeldahl method (27).

**Preparation of the liver and enzyme activity assay**

Liver mitochondrial and cytosol fractions were prepared as described in our previous report (28). Carnitine palmitoyltransferase-2 (CPT2, EC 2.3.1.21) (29) activity in the mitochondrial fraction and fatty acid synthase (FAS, EC 2.3.1.85) (30), acetyl-coenzyme A carboxylase (ACC, EC 6.4.1.2) (31), malic enzyme (ME, EC 1.1.1.40) (32), and glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) (33) activities in the cytosol fraction were assayed spectrophotometrically. The protein contents of mitochondrial and cytosol fractions were determined according to the method of Lowry et al. (34).

**Statistical analysis**

Data are presented as mean and standard deviation (SD). For the *in vitro* study, comparisons between casein and EPL digests were performed by Student's *t*-test. For the *in vivo* study, comparisons among the three groups were performed by one-way ANOVA followed by the Tukey-Kramer test. Differences were defined as significant at $P<0.05$. Analyses were performed using StatView-J version 5.0 software (Abacus Concepts, Inc., Berkeley, CA, USA).

## RESULTS AND DISCUSSION

EPL has been used as a food preservation substrate (17) and has anti-microbial (16) and anti-viral activities (18). In addition, EPL also has a hypotriacylglycerolemic effect through the inhibition of pancreatic lipase activity (13, 21). We hypothesized that EPL may play a contributing role to the inhibition of neutral and acidic sterol absorption. To clarify this hypothesis, we, for the first time, reported that EPL has the effects of decreasing micellar lipid solubility *in vitro* and suppressing neutral and acidic sterol absorption *in vivo*.

Protein is digested by proteases including pepsin, trypsin, chymotrypsin, and elastase in the gastrointestinal tract. Protein digests showed different statuses, such as in terms of the molecular weight and conformation, dependent on the duration of digestion. Therefore, it is not appropriate to use intact protein to assess bile acid binding capacity, lipase inhibition activity, and micellar lipid solubility. This study examined the production of casein and EPL digests prepared using a simulated digestion model (3). The effects of bile acid binding capacity, lipase inhibition activity, and micellar lipid solubility were measured in the casein and EPL digests after the addition of pancreatin because the degradation and absorption of lipids, including TG, phospholipid, fatty acid, cholesterol, and bile acid occur in the intestine. In addition, we also determined the physiological properties of L-lysine, which is an EPL component.

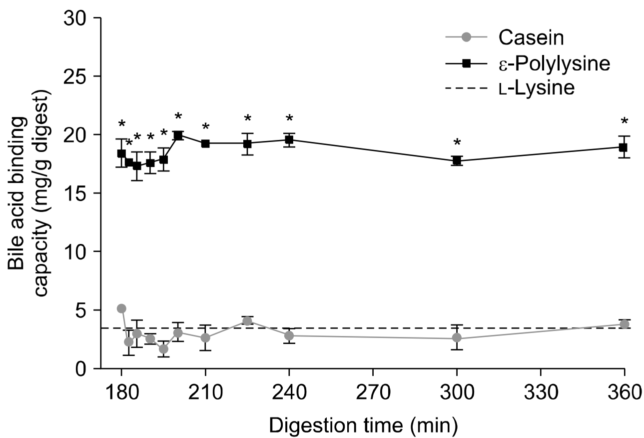


Fig. 1. Course of bile acid binding capacity in casein and ε-polylysine digests prepared by simulated gastrointestinal digestion. Each point represents mean±SD (n=3). Dotted line indicates means of L-lysine (n=3). Asterisks indicate statistically significant differences from casein at $P<0.05$ at the same time point. Data were analyzed by Student's $t$-test.

Fig. 1 shows the time courses of bile acid binding capacity for casein digest, EPL digest, and L-lysine. Upon comparisons at the same time points, the EPL digest had significantly higher bile acid binding capacity than the casein digest at all time points. In addition, the bile acid binding capacities of casein digest and L-lysine were maintained at around 4 mg/g. EPL is synthesized by linking the ε-amino group with its α-carboxyl group, and it is not hydrolyzed by proteases (16). In this study, EPL contained 44.6% (wt/wt) dextrin and its dextrin could be hydrolyzed by pancreatin, which has not only protease activity but also amylase activity. From these results, it was speculated that the dextrin in EPL did not affect the bile acid binding capacity. Protamine hydrochloride, which belongs to the class of basic proteins, bound to bile acid and formed an insoluble complex instantly (11). In this study, it was confirmed visually that the insoluble complex was generated when EPL digest and bile acid in bile were mixed (data not shown). Therefore, EPL digest coupled with bile acid and formed an insoluble complex.

Fig. 2 shows the pancreatic lipase (A) and cholesterol esterase (B) activities in the presence of casein digest, EPL digest, and L-lysine. EPL digest was strongly in-

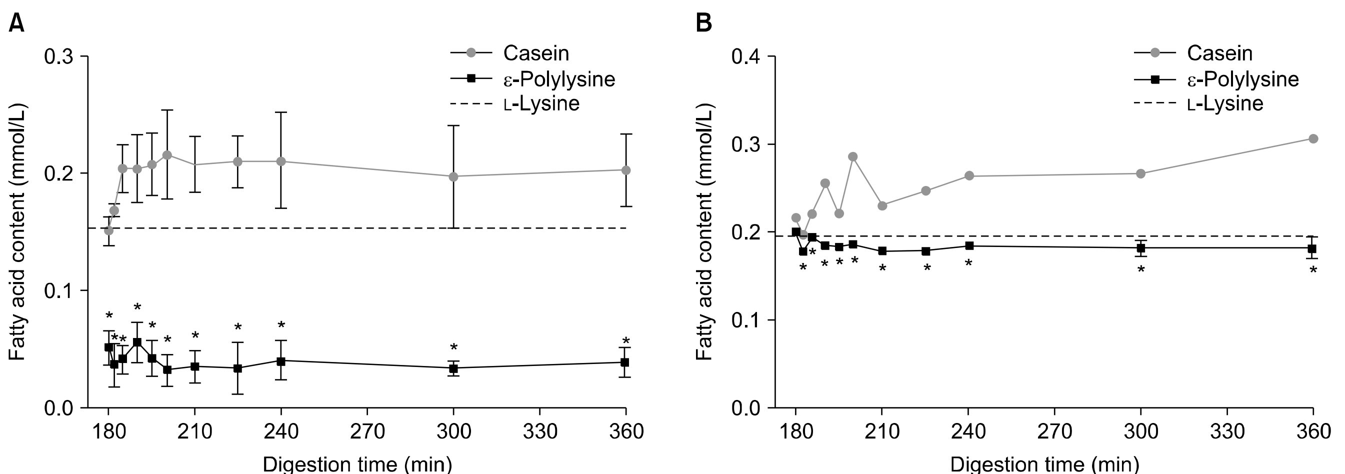


Fig. 2. Course of lipase (A) and cholesterol esterase (B) activities in the presence of casein and ε-polylysine digests at a concentration of 1 g/L. Each point represents mean±SD (n=3). Dotted line indicates means of L-lysine (n=3). Asterisks indicate statistically significant differences from casein at $P<0.05$ at the same time point. Data were analyzed by Student's $t$-test.

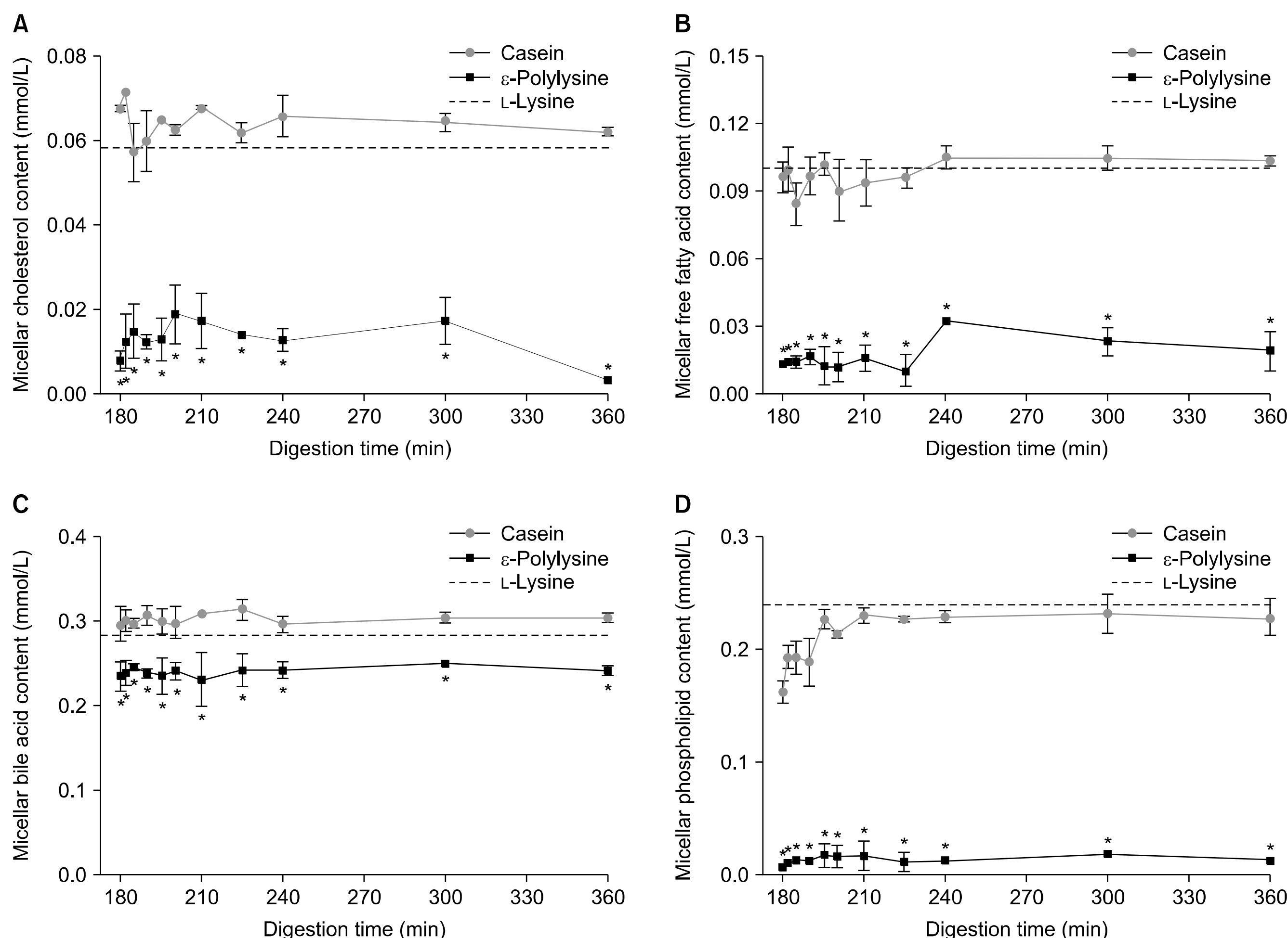


**Fig. 3.** Course of micellar cholesterol (A), free fatty acid (B), bile acid (C), and phospholipid (D) contents in the presence of casein and ε-polylysine digests. Each point represents mean±SD (n=3). Dotted line indicates means of L-lysine (n=3). Asterisks indicate statistically significant differences from casein at $P<0.05$ at the same time point. Data were analyzed by Student's *t*-test.

hibited in terms of the activity of pancreatic lipase compared with the casein digest at all time points. Cholesterol esterase activity was weakly inhibited by the EPL digest. These findings support the results of the inhibition of lipase and esterase activities in the presence of intact EPL in previous studies (12,14). In addition, other research showed that the intact EPL suppressed pancreatic lipase activity in a manner dependent on the breakdown of bile acid emulsion (13). This study confirmed that the EPL digest has strongly bile acid binding capacity and formed an insoluble complex as described above. Hence, the EPL digest inhibited pancreatic lipase and cholesterol esterase activities by breaking down the bile acid emulsion and forming an insoluble bile acid-EPL complex.

After the lipid digestion, cholesterol, monoglyceride, and fatty acid associated with bile acid and phospholipid form a micelle. A micelle is necessary for absorption because it transports the poorly soluble cholesterol, monoglyceride, and fatty acid to the surface of the enterocyte (35). Fig. 3 shows the solubility of micellar cholesterol, free fatty acid, bile acid, and phospholipid in the presence of casein digest, EPL digest, and L-lysine. Upon comparisons at the same time points, the EPL digest had significantly lower micellar cholesterol, free fatty acid, bile acid, and phospholipid solubility than the casein digest at all time points. These results suggest that the EPL digest could inhibit lipid absorption in the digestive tract through suppressing the micellar lipid solubility. In the second experiment, male Wistar rats were fed a diet containing 1% (wt/wt) EPL or L-lysine for 4 weeks, after which the effect of dietary EPL was examined. EPL has been used as a food preservative at levels of 10～500 ppm in boiled rice, noodle soup stocks, other soups, noodles and cooked vegetables (17). In this study, the EPL diet was adjusted to approximately 500 mg/kg body weight (500 ppm), which is the maximum amount contained in food.

Table 2 shows growth parameters, organ weights, and lipid contents in serum and liver. No differences were observed in body weight, body weight gain, energy intake, and food efficiency among the groups. In addition, there were no significant differences in relative liver weights, epididymal WAT, mesentery WAT, perirenal

**Table 2.** Effect of dietary ε-polylysine and L-lysine on growth parameters, organ weights, and serum and liver lipid contents in rats

| | Dietary group | | |
|---|---|---|---|
| | Control | EPL | LYS-AA |
| Growth parameter | | | |
| Initial BW (g) | 122±7 | 122±5 | 122±7 |
| Final BW (g) | 312±6 | 309±12 | 314±16 |
| BW gain (g/d) | 6.79±0.20 | 6.68±0.47 | 6.85±0.48 |
| Energy intake (kcal/d) | 70.5±7.2 | 72.8±8.6 | 72.4±7.4 |
| Food efficiency (g/kcal)[1)] | 0.096±0.003 | 0.092±0.006 | 0.095±0.007 |
| Organ weight (g/100 g BW) | | | |
| Liver | 3.56±0.10 | 3.51±0.13 | 3.68±0.18 |
| Epididymal WAT | 1.56±0.19 | 1.44±0.23 | 1.67±0.14 |
| Mesentery WAT | 1.55±0.33 | 1.31±0.41 | 1.44±0.13 |
| Perirenal WAT | 1.09±0.16 | 1.02±0.15 | 1.02±0.06 |
| Total WAT[2)] | 4.20±0.64 | 3.76±0.76 | 4.12±0.27 |
| Serum (mg/dL) | | | |
| Phospholipid | 142±9[a] | 121±11[b] | 126±19[ab] |
| TG | 51.6±16.3[a] | 34.3±3.4[b] | 41.3±8.1[ab] |
| Cholesterol | 75.9±5.5[a] | 63.7±10.1[b] | 68.1±10.5[ab] |
| HDL cholesterol | 49.7±5.1 | 40.4±8.1 | 43.0±7.6 |
| Non-HDL cholesterol | 26.1±1.2[a] | 22.5±1.6[b] | 25.1±3.7[ab] |
| Liver (mg/g liver) | | | |
| Phospholipid | 23.6±4.5 | 24.4±1.2 | 23.9±0.6 |
| TG | 44.0±7.5[a] | 26.6±12.9[b] | 38.9±13.5[a] |
| Cholesterol | 3.98±1.29[a] | 2.46±0.66[b] | 4.04±0.63[a] |

Data are presented as mean±SD (n=7). Values not sharing a common letter are significantly different at $P<0.05$. Data were analyzed by Tukey-Kramer test.
[1)]Food efficiency=BW gain (g/d)/energy intake (kcal/d).
[2)]Total WAT=epididymal WAT+mesentery WAT+perirenal WAT.
BW, body weight; EPL, ε-polylysine; LYS-AA, L-lysine-amino acid; WAT, white adipose tissue; TG, triacylglycerol; HDL, high-density-lipoprotein.

WAT, and total WAT weights among the groups. The EPL group exhibited significantly lower serum phospholipid, TG, cholesterol, and non-HDL cholesterol contents than the control group, but no significant difference was observed between the EPL and the LYS-AA groups. Although there was no significant difference in the *in vitro* experiment between casein and L-lysine levels, the dietary LYS-AA diet tended to have lower serum lipid contents than the control diet. The possible mechanism behind this might be the physiological function of L-lysine, such as its fundamental role in the production of carnitine, which shuttles long-chain fatty acids into the mitochondria for energy production (36). Rats have higher serum HDL cholesterol content than rabbits and humans because they are naturally deficient in cholesteryl ester transfer protein activity (37). Therefore, the decrease of cholesterol content by the EPL diet was confirmed in the serum HDL fraction. In addition, liver TG and cholesterol contents in the EPL group were significantly lower than those in the control and the LYS-AA groups. The decreases in TG contents in serum and liver were assumed to be mainly due to the inhibition of pancreatic lipase activity and a reflection of endogenous fatty acid metabolism, including the suppression of fatty acid synthesis and the enhancement of fatty acid β-oxidation (15). Dietary EPL has been reported to decrease serum TG content through the inhibition of lipase activity in rats and mice (12,13). In this study, we also found the reduction of serum and liver TG contents, partly due to the enhancement of fecal fatty acid excretion (Table 3), which is related to the inhibition of lipase activity by the EPL digest (Fig. 2). Fig. 4 shows the enzyme activities of fatty acid biosynthesis in the liver. Interestingly, the EPL diet had significantly higher activities of G6PDH and ACC, which are key enzymes in fatty acid biosynthesis, than the control diet. The activity of FAS, a rate-limiting enzyme in fatty acid synthesis, in the EPL group tended to be higher than in the control group. However, the activity of CPT2, which is a mitochondrial fatty acid oxidation enzyme, was not influenced by the consumption of the EPL and the LYS-AA diets (data not shown). The enhancement of fatty acid biosynthesis in the liver due to dietary EPL contradicts the reduction in the serum and liver lipid contents. We thus hypothesized that the decrease in serum and liver TG contents with the EPL diet was attributable to the inhibition of TG absorption in the intestine and not to the influence of *de novo* fatty acid synthesis.

**Table 3.** Effect of dietary ε-polylysine and L-lysine on fecal weight and lipid excretion in rats

| | Dietary group | | |
|---|---|---|---|
| | Control | EPL | LYS-AA |
| Fecal weight (g/d group) | $23.1\pm2.9^{b}$ | $33.1\pm2.3^{a}$ | $28.0\pm6.2^{ab}$ |
| Fatty acid (mg/d group) | $4.04\pm0.89^{b}$ | $21.35\pm3.25^{a}$ | $5.28\pm1.16^{b}$ |
| Neutral sterol (μmol/d group)[1)] | $69.3\pm12.0^{b}$ | $227.0\pm35.8^{a}$ | $93.4\pm21.9^{b}$ |
|   Cholesterol (μmol/d group) | $13.6\pm2.5^{b}$ | $34.7\pm17.4^{a}$ | $18.7\pm3.9^{b}$ |
|   Coprostanol (μmol/d group) | $55.7\pm9.8^{b}$ | $192.4\pm25.4^{a}$ | $74.7\pm18.3^{b}$ |
| Acidic sterol (μmol/d group) | $72.3\pm7.1^{c}$ | $183.8\pm14.2^{a}$ | $111.8\pm30.6^{b}$ |
| Total sterol (μmol/d group)[2)] | $141.6\pm13.5^{c}$ | $410.9\pm42.7^{a}$ | $205.2\pm45.0^{b}$ |
| Nitrogen (mg/d group) | $161\pm8^{b}$ | $220\pm52^{a}$ | $207\pm30^{ab}$ |

Data are presented as mean±SD (n=7). Values not sharing a common letter are significantly different at $P<0.05$. Data were analyzed by Tukey-Kramer test.
[1)]Neutral sterol=cholesterol+coprostanol. [2)]Total sterol=neutral sterol+acidic sterol.
EPL, ε-polylysine; LYS-AA, L-lysine-amino acid.

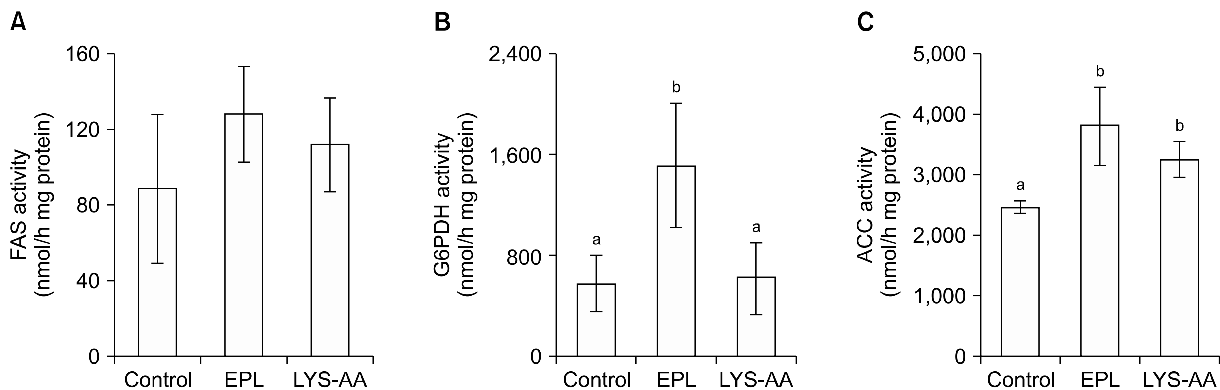


**Fig. 4.** The activities of FAS (A), G6PDH (B), and ACC (C) related to the fatty acid metabolic pathway in the liver. Data are mean±SD (n=7). Values not sharing a common letter are significantly different at $P<0.05$. Data were analyzed by Tukey-Kramer test. FAS, fatty acid synthase; G6PDH, glucose-6-phosphate dehydrogenase; ACC, acetyl-coenzyme A carboxylase; EPL, ε-polylysine; LYS-AA, L-lysine-amino acid.

The increased activities of fatty acid biosynthesis due to dietary EPL may be prevented by the enhancement of fecal lipid excretion. Our previous study showed that dietary protamine simultaneously enhanced hepatic FAS activity and fecal fatty acid excretion (27). Compared with high-fat feeding, low-fat feeding has been suggested to increase the mRNA expression and enzyme activities for fatty acid synthesis in animal models (38,39). Therefore, the elevated activity of fatty acid synthesis-related enzymes in the EPL group could have been caused by the interruption of fatty acid absorption in the intestine. The expression of FAS and ACC is regulated by sterol regulatory element-binding protein-1c and liver-X receptor, which are a transcriptional factor and a nuclear receptor (40,41). Further studies are necessary to clarify the effects of dietary EPL on the expression of transcriptional factors and nuclear receptors.

Table 3 shows the fecal lipid and nitrogen excretion. The EPL group had higher fecal weight, fatty acid, neutral sterol, including cholesterol and coprostanol, acidic sterol, and total sterol than the control and the LYS-AA groups. The reduction of serum cholesterol content due to several dietary proteins compared with that of casein was found to depend on their amino acid composition and the digestive tract effects of their digestion products (3,42). One of these factors is the amino acid composition of the protein, in particular the ratio of lysine/arginine (43), and its content of specific amino acids, namely, methionine (44), cysteine (45), and glycine (46). In this study, however, the amino acid composition of the EPL diet proved to be similar to that of the LYS-AA diet, so the difference could not be explained in terms of differences in lysine/arginine, methionine, cysteine, and glycine contents. This suggests that the cholesterol-lowering effect of EPL was not the result of metabolic function due to differences in amino acid composition.

Another of the relevant factors involves digestive tract effects, which assumes that the digestibility of dietary proteins and the physicochemical properties of digestion products in the digestive tract are related to cholesterol metabolism (6). Nagata et al. reported that serum cholesterol-lowering activity depends on the extent of fecal neutral and acidic sterol excretion (5). To clarify the mechanism underlying the cholesterol-lowering activity that is associated with EPL, we analyzed fecal neutral sterol, acidic sterol, and nitrogen excretion. Rats fed the

EPL diet had significantly higher fecal neutral sterol, acidic sterol, total sterol, and nitrogen contents than rats fed control and the LYS-AA diets (Table 3). Many researchers have demonstrated that the enhancement of fecal neutral and acidic sterol excretion is accompanied by an increase in fecal nitrogen excretion (7,8). EPL is resistant to proteases in the gastrointestinal tract, as described previously (13). The enhancement of fecal sterol excretion by EPL was at least partly due to the indigestibility because fecal nitrogen excretion was increased. The hypocholesterolemic effects induced by the increase of fecal sterol excretion were reported to show suppressed micellar cholesterol solubility and high bile acid binding capacity *in vitro* (3,4). Dietary EPL exhibited decreased serum and liver cholesterol contents compared with the control and the LYS-AA diets, owing to the suppression of neutral and acidic sterol absorption through decreasing micellar cholesterol solubility and the high bile acid binding capacity of EPL. The LYS-AA group did not show decreased serum and liver cholesterol contents or increased fecal neutral sterol excretion. The inhibition of sterol absorption has been reported for peptides with a heptapeptide size or greater (9,10). The enhancement of fecal sterol excretion by dietary EPL is not only physiologically associated with L-lysine; peptide length has a more important role. In this study, we confirmed that EPL has high bile acid binding capacity and formed an insoluble complex with bile acid (Fig. 1). Bile acid is amphipathic, that is, it contains both hydrophobic and hydrophilic regions. Previous studies suggested that the insoluble high-molecular-weight peptide fraction, which is rich in hydrophobic amino acids, could bind to bile acid by hydrophobic interactions and inhibit bile acid reabsorption (47,48). EPL has low hydrophobicity, so it did not combine with bile acids by hydrophobic interactions. EPL binds to some proteins, nucleic acids, viruses, or bacteria through electrostatic interactions and inhibits their functions (16). Therefore, the hypocholesterolemic effect of EPL might occur through its binding to bile acids and phospholipids by electrostatic interactions and the inhibition of intestinal neutral and acidic sterol absorption. In addition, in order to bind to bile acids and phospholipids by electrostatic interaction, L-lysine needs to be in peptide form.

## CONCLUSION

The present study revealed that, compared with dietary casein and L-lysine, dietary EPL decreased serum and liver lipid contents through the enhancement of fecal fatty acid and neutral/acidic sterol excretion. New findings in the present study were that the inhibition of sterol absorption due to EPL was mediated by the suppression of micellar cholesterol solubility and high bile acid-binding capacity. In addition, EPL enhanced the activities of enzymes related to fatty acid biosynthesis in the liver. This study found that EPL has a hypolipidemic effect, which would be beneficial in the prevention of hyperlipidemia, obesity, and arteriosclerosis.

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## AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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