Title: **Porcine artery elastin preparation reduces serum cholesterol level in rats**

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Running title: Porcine artery elastin reduces serum cholesterol

Abstract

The effect of porcine artery elastin on serum cholesterol level was investigated in rats fed a cholesterol-free diet. Rats were fed for 4 weeks, with a diet (ED) containing 15% casein and 5% of porcine artery elastin in comparison with a diet (CD) containing 20% casein. The total serum and non-HDL-cholesterol concentrations were lower ($P<0.001$) in ED-fed group than the CD-fed group at the end of the experiment. Caecal propionate concentration and *Bifidobacterium* and *Lactobacillus* population were higher ($P<0.05$) in ED-fed group than the CD-fed group. The results of this study suggest that porcine artery elastin could be considered as a functional dietary protein with hypocholesterolaemic ability. Favourable amino acid composition and lysine derived cross links may at least be partially responsible for the hypocholesterolaemic ability of ED. Moreover, the higher caecal propionic acid concentration in the ED-fed group may have suppressed the cholesterol synthesis in the liver, and reduced the serum cholesterol level.

1. Introduction

In recent years, efforts have been made to introduce additional physiologically functional properties into meat products (Fernandez et al., 2005; Jimenez et al., 2001; Arihara, 2004). Utilization of functional ingredients is one approach for the development of functional meat products. From previous studies it has been shown that pork peptides have antithrombotic and antioxidative effects (Shimizu et al., 2009; Saiga et al., 2003). Furthermore, the antioxidative activities of pepsin-solubilized elastin and acid-solubilized elastin have been reported (Hattori et al., 1998). The influence of elastin peptides on oxygen metabolism may be related to their activities *in vivo* following elastin degradation and can contribute to their role in pathogenesis of atherosclerosis (Weglarz et al., 1992). Although, the bioactive proteins such as hypocholesterolaemic agents have not been utilized in the meat industry, meat products with such activity could open up a new market. There are still some hurdles in developing and marketing novel functional meat products since such products are unconventional and consumers in many countries recognise meat and meat products to be bad for health. Along with accumulation of scientific data, there is an urgent need to inform consumers of the exact functional value of meat and meat products including novel functional foods. Elastin used in this study was extracted from porcine arteries, which are waste products of the porcine industry. Elastin is also primarily composed of the some favourable amino acids glycine, valine, alanine, and proline, which could play a considerable effect on lipid metabolism (Sandberg et al.,1981). This hypothesis may be supported by previous findings showing that dietary proteins with favourable amino acid composition reduced the serum cholesterol level in rats (Morita et al., 1997; Gudbrandsen et al., 2005). The other characteristic feature of the amino acid composition of porcine elastin preparation is the presence of lysine derived cross links: desmosine and isodesmosine as described previously by Thomas et al. (1963). In this study we investigated the effect of porcine artery elastin preparation on lipid metabolism in rats fed on a cholesterol-free diet.

2. Materials and methods

2.1. Animals and diets

Seven weeks old male Fischer rats (162.08± 4.62 g) were purchased from Charles River Japan Inc. (Yokohama, Japan). They were housed individually in cages with free access to food and water. The animal facility was maintained on a 12 h light/dark cycle at a temperature of 23 ± 1 ºC and relative humidity of 60 ± 5 %. The rats were randomly assigned into two groups (n=5). The composition of each diet is shown in Table 1. The experimental rats were fed for 4 weeks, with 20% casein diet (CD), in comparison with diet containing 15% casein and 5% of elastin (ED). The elastin protein preparation "P-elastin" was a kind gift from the Nippon Meat Packers, Inc. (Tsukuba, Japan). Amino acid composition of the “P-elastin” is shown in Table 2, which is similar to that of pig (Foster et al.1980). The rats were allowed free access to food and water for 4 weeks. Body weight and food consumption were recorded weekly and daily, respectively. This experimental design was approved by the Animal Experiment Committee of Obihiro University of Agriculture and Veterinary Medicine. All animal procedures conformed to standard principles described in Guide for the care and Use of Laboratory Animals (National research council, Washington DC,1985).

2.2. Analytical procedures

Blood samples (1 ml) were collected between 08.00 and 10.00 h from the jugular vein of fasting rats anaesthetised by sodium pentobarbital. The samples were taken into tubes without an anticoagulant. After the samples were allowed to stand at room temperature for 2 h, the sera were separated by centrifugation at 1500 g for 20 min. All faecal excretions were collected during the last 3 days of the experimental period (4 weeks). At the end of the experiment, the rats were anaesthetised with sodium pentobarbital and killed. The livers and caecum were quickly removed, washed with cold saline (9 g NaCl/l), blotted dry on filter paper, and weighed before freezing for storage.

2.3. Chemical analysis

Total cholesterol (TC), HDL-cholesterol (HDL-C), and triacylglycerol (TG) concentrations in the serum were determined enzymatically using commercially available reagent kits (assay kits for the TDX system; Abbott laboratory Co., Irving, TX). The non-HDL-cholesterol concentration was calculated as follows: non-HDL-cholesterol = total cholesterol - HDL-cholesterol. Total lipids were extracted from liver and faeces by a mixture of chloroform/methanol (2:1, v/v) (Folch et al., 1957). The neutral steroids in each lipid sample obtained by saponification were acetylated (Matsubara et al.,1990) and analyzed by gas–liquid chromatography (GLC) using a Shimadzu 14A chromatograph (Kyoto, Japan) with a DB17 capillary column (0.25 mm×30 m; J&W Scientific, Folsom, CA) with nitrogen as the carrier gas. Acidic sterols in faeces were measured by GLC following the method of Grundy et al. (1965). A part of the caecal content was taken out into desalting water in a vial without exposure to air, and suspended. The suspension of caecum was deproteinized with perchloric acid and to form sodium salts of the short chain fatty acids (SCFAs). Individual SCFAs were measured by GLC using a glass column (2000 x 3 mm) packed with 80–100 mesh chromosorb W-AW DMCS with H_3PO_4 (100 ml/l) as the liquid phase after adding H_3PO_4 by the procedure of Hara et al. (1994).

2.4. RNA isolation, RT-PCR and Southern blot analysis

RNA isolation, semi-quantitative RT-PCR and Southern blot analysis were done as described previously (Chomczynski and Sacchi, 1987, Han et al.2005, Ruvini et al.2007). The mRNAs encoding the LDL receptor , cholesterol 7α-hydroxylase , 5-hydroxy-3-methylglutaryl-coenzyme A reductase , sterol regulatory elementary binding protein -1c, fatty acid synthase , and GAPDH (used as an invariant control) were analyzed.

2.5. Growth of bacteria in the caecum

Coliform in the caecum was inoculated and grown for 2 days on DHL agar (Eiken

Chemical Co., Ltd, Tokyo, Japan) plates at 37 °C. Anaerobe, *Lactobacillus* and *Bifidobacterium* in the caecum were incubated for 5 days on GAM agar (Nissui Pharmaceutical Co., Ltd, Tokyo, Japan), Rogosa agar (Merck KGaA, Darmstadt, Germany) and BL agar (Eiken Chemical Co., Ltd, Tokyo, Japan) at 37 °C by the gaspak method according to the procedure of Mitsuoka et al. (1964, 1965, 1976).

2.6. Statistical analysis

Data are presented as means ± SD for five rats. The significance of difference between two groups was determined by student's t-test. Difference was considered significant at $P<0.05$, and $P<0.001$.

4. Results and discussion

In this study, we examined the effect of porcine artery elastin preparation on serum lipids, liver cholesterol, faecal lipids, caecal lipids, caecal bacterial population, and hepatic mRNAs in rats. There was no significant difference in the food intake, body weight gain, liver weight, caecal weight, faecal dry weight and hepatic mRNAs between two groups at the end of the experimental period (data not shown). The lower serum TC and non-HDL-C level in ED-fed group (Table 3) at the end of the 4-week feeding period suggest that elastin may have acted as a functional protein and modulated the cholesterol metabolism in rats. However, serum TC, non-HDL, and HDL-C concentration were lower ($P<0.05$) at the beginning of the experiment (Table 3). The reason for the different serum cholesterol levels at week 0 may simply be the current variability between animals. Feeding ED for 4-weeks reduced the TC and non-HDL-C levels to the extent that they were significantly different at $P<0.001$. Moreover, feeding ED for 4-weeks eliminated the difference in HDL-C level observed at the 0-week. Lower serum cholesterol level in ED-fed group may be supported by the favourable amino acid composition as shown previously for some other dietary proteins in rats (Morita et al., 1997; Gudbrandsen et al., 2005). Elastin is known to have a highly distinctive amino acid composition, especially

very low methionine to glycine ratio, may be one of the major reasons for lower serum cholesterol level in this study. Lower methionine level or lower methionine to glycine ratio in elastin might have hindered the transfer of cholesterol from liver into blood stream (Morita et al., 1997). In fact, the lower methionine level may reduce phosphatidylcholine synthesis via phosphatidylethanolamine, leading to depression of apolipoprotein release into circulation (Morita et al., 1997). The lower lysine to arginine ratio in elastin compared to casein may be the reason for lower atherogenic index which is considered to be a risk indicator for cardiovascular diseases (Kritchevsky et al., 1982). Moreover, the presence of lysine derived cross links: desmosine and isodesmosine may at least be partially responsible for hypocholesterolaemic ability of porcine artery elastin. Other than amino acid composition, elastin peptides or specific peptide fragments released during the elastin digestion may also be partially responsible for the observed effects as suggested previously (Korhonen and Pihlanto, 2003).

The faecal coprostanol and neutral sterol concentration was lower ($P<0.05$) in ED-fed group than that in the CD-fed group at the end of the 4-week experimental period (Table 4). Lower serum cholesterol level in ED-fed group was not supported by faecal sterol excretion and hepatic mRNA expression. However, the higher propionic acid concentration in the caecum (Table 4) may at least be partially responsible for the lower serum cholesterol level in ED-fed group as shown previously (Anderson and Bridges, 1984; Nishina and Freedland, 1990). Higher caecal propionic acid concentration in ED-fed group was further supported by the lower ($P<0.05$) caecal pH [6.71±0.16(ED), 7.18±0.28(CD)] and higher *Bifidobacterium* and *Lactobacillus* population (Table 5). It was suggested that protein was one of the major substrates for caecal fermentation in rats when animal protein based diet was fed (Tsukahara and Ushida, 2000).

In conclusion, elastin could be considered as a functional dietary protein with hypocholesterolaemic ability. Favourable amino acid composition and lysine derived cross links may at least be partially responsible for the hypocholesterolaemic ability of porcine artery elastin. Moreover, the elastin have induced caecal fermentation and resulting higher caecal propionic acid concentration may have suppressed the cholesterol synthesis in the liver, and reduced the serum cholesterol level.

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Appendix

CD; casein diet, ED; porcine artery elastin diet, TC; total cholesterol, HDL-C; High density lipoprotein cholesterol, TG; Triacylglycerol, SCFA; short chain fatty acid

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Table 1. Composition of experimental diets

Ingredients	Dietary group[a]	
	CD	ED
	g/kg diet	
Casein	200	150
Elastin	-	50
Soybean oil	70	70
Mineral mixture[b]	35	35
Vitamin mixture[c]	10	10
Cellulose powder	50	50
Sucrose	100	100
L-cystine	3	3
Choline hydrogen tartrate	2.5	2.5
3-Butylhydroquinone	0.014	0.014
α-Corn starch	529.486	529.486

[a]CD, Casein diet; ED, Elastin diet

[b]AIN-93G mineral mixture

[c]AIN-93G vitamin mixture

Table 2. Amino acid composition of porcine artery elastin preparation

Amino acids	Volume basis (v/v%)	Weight basis (w/w%)
Hydroxyproline	0.80	1.07
Aspartic acid	0.61	0.83
Threonine	1.14	1.37
Serine	0.96	0.99
Glutamic acid	2.14	3.28
Proline	11.11	12.80
Glycine	33.52	22.69
Alanine	23.09	19.47
Valine	12.41	14.59
Methionine	0.04	0.07
Isoleucine	1.81	2.43
Leucine	5.74	7.70
Tyrosine	1.72	3.34
Phenylalanine	3.15	5.51
Lysine	0.88	1.33
Arginine	0.68	1.26
Isodesmosine	0.08	0.53
Desmosine	0.12	0.74

Table 3. Serum total cholesterol, non-HDL- cholesterol, HDL-cholesterol, triacylglycerol concentrations and atherogenic index in rats fed experimental diets for 4 weeks

Dietary group	Wk0	Wk2	Wk4
		mmol/l	
Total cholesterol			
CD	1.79±0.13	1.70±0.15	2.08±0.09
ED	1.57±0.12 *	1.54±0.10	1.72±0.13 * *
HDL-cholesterol			
CD	0.80±0.06	0.55±0.02	0.72±0.03
ED	0.72±0.04 *	0.57±0.04	0.70±0.05
non -HDL-cholesterol			
CD	0.99±0.09	1.14±0.14	1.36±0.08
ED	0.85±0.09 *	0.97±0.09	1.02±0.10 * *
Triacylglycerol			
CD	0.68±0.10	1.17±0.25	1.28±0.27
ED	0.58±0.14	0.84±0.27	1.04±0.35
Atherogenic index[a]			
CD	1.25±0.10	2.06±0.23	1.94±0.26
ED	1.18±0.09	1.71±0.20 *	1.43±0.10 *

Values are expressed as means ± SD for five rats. *$P < 0.05$ vs. control by student's *t*-test.

•* $P < 0.001$ vs. control by student's *t*-test. CD, Casein diet; ED, Elastin diet.

•[a] Atherogenic index =(LDL-cholesterol/HDL-cholesterol)

Table 4. Faecal neutral steroid and caecal short chain fatty acid concentrations in rats fed experimental diets for 4 weeks

	Dietary group	
	CD	ED
Faecal lipids		
Faecal cholesterol (μmol/faeces 1g)	4.96±0.99	5.05±1.25
Faecal coprostanol (μmol/faeces 1g)	12.76±3.99	5.28±3.81 *
Faecal neutral sterol (μmol/faeces 1g)	17.72±4.62	10.32±4.22 *
Caecal lipids		
Caecal acetate(μmol/rat)	0.052±0.015	0.095±0.049
Caecal propionate(μmol/rat)	0.021±0.009	0.035±0.008 *
Caecal butyrate(μmol/rat)	0.017±0.007	0.020±0.005
Caecal SCFA(μmol/rat)	0.090±0.030	0.149±0.058

Values are expressed as means ± SD for five rats. *$P < 0.05$ vs. control by student's *t*-test.

SCFA; short chain fatty acids; CD, Casein diet; ED, Elastin diet

Table 5. Caecal bacterial population in rats fed experimental diets for 4 weeks

Dietary group	CD	ED
	log10 cfu/g wet caecum	
Bifidobacterium	4.74±1.21	6.15±0.41*****
Lactobacillus	4.19±0.66	5.57±0.41*****
Anaerobe	6.27±0.06	7.44±0.28

Values are expressed as means ± SD for five rats.

**P* < 0.05 vs. control by student's *t*-test.

CD, Casein diet; ED, Elastin diet.