Title : Preparation method modulates hypocholesterolaemic responses of potato peptides

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Running title: hypocholesterolaemic responses of potato peptides

Abstract

In this study we compared the hypocholesterolaemic ability of two potato peptide preparations in rats. Experimental groups were fed for 4 weeks, with casein as the basal diet, in comparison with two diets containing 20% potato peptide preparations PPS (Short hydrolysis preparation) and PPL (Long hydrolysis preparation). Serum total cholesterol and serum triglyceride level were lower in PPS-fed group compared with CN-and PPL-fed groups. Lower non-HDL cholesterol level (*P*<0.05) in both PPS-and PPL-fed groups, was followed by higher neutral sterol excretion, and higher hepatic LDL-R and SR-B1 mRNA level than the control. Hepatic SREBP-2 and HMG-CoA reductase mRNA level were higher in PPL-fed group compared with the CN-fed group (*P*<0.05). Caecal total SCFA concentration was higher in PPL-fed group relative to PPS-and CN- fed groups. Based on these data, it could be suggested that the difference in the preparation method may modulate the hypocholesterolaemic responses of potato peptides in rats.

KEY WORDS: Potato peptide; Cholesterol; Short chain fatty acid; Faecal steroid; Hepatic gene; Rat.

1. Introduction

In our previous studies, it was observed that potato peptides have the ability to alter serum lipids when rats fed either on a cholesterol-free diet (Liyanage et al., 2008) or cholesterol enriched diet (Liyanage et al., 2009). However, to reduce the cost of production we altered the preparation method in order to obtain a higher recovery percentage. In this study, we investigated and compared the ability of altered potato peptide preparation (PPL) to modulate the lipid metabolism in rats, in comparison with the previous preparation (PPS). Potato peptide mixtures used in this study produced by enzymatic hydrolysis method using commercial enzymes and different from each other due to the length of hydrolysation. From previous findings it has been shown that, depending on the initial protein source, enzyme used, and processing conditions, the biological activities of the peptides are different (Kim et al., 2000; Pena-Ramos and Xiong, 2002; Wu et al., 2001). Length of hydrolysis may alter the degree of hydrolysation, by increasing the number of free amino acids or reducing the number of peptide bonds. Recovery percentage of the long hydrolysis preparation (PPL) was about 40% which was greater than that (25%) in short hydrolysis preparation (PPS) in previous experiments (Liyanage et al., 2008; Liyanage et al., 2009).

From previous studies it has been shown that the variability in cholesterolaemic responses to different soy-based diets in clinical studies is related to the specific protein composition of the soy variant used (Gianazza et al., 2003). Further, it has been suggested that protein-induced alterations of cholesterol metabolism may be mediated by differences in the amino acid patterns of dietary proteins or by bioactive peptides (Gudbrandsen et al., 2005; Lovati et al., 2000; Sugiyama et al., 1996). Thus, the differences in two peptide preparations, causing substrate difference, may contribute to the differences in the lipid metabolism in the present study.

Besides the concentration of serum lipids, liver cholesterol, faecal lipids, and caecal lipids, we determined the relative mRNA concentrations of genes related to cholesterol metabolism in this study. Moreover, we measured the hepatic SREBP-2 concentration, which is the transcriptional factor for genes involved in cholesterol uptake and biosynthesis, such as hydroxymethyl glutaryl-CoA reductase and the LDL receptor, (Amemiyo-Kudo et al., 2002) to clarify the gene nutrient interaction in cholesterol metabolism of this study.

2. Materials and methods

2.1. Animals and diets

Fifteen male Fischer-344 rats (7-weeks old) were randomly assigned to three groups of 5 each (Charles River, Yokohama, Japan). All rats were individually housed in plastic cages. 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 composition of each diet is casein,200; L-Cystine,3;Soybean oil,50; Mineral Mixture,35;Vitamin mixture,10; Choline bitartrate,2.5; Tert-Butyl hydroquinone, 0.014; cellulose powder,50; sucrose,100; acorn starch,549.486; shown in Table 1. The experimental groups were fed for 4 weeks, with casein as the basal diet, in comparison with two diets containing 20% potato peptide preparations (PPS and PPL). The rats were allowed free access to food and water for 4-week experimental period. Body weight and food consumption were recorded weekly and daily, respectively. The blood samples (1 mL) were taken every week between 09.00 and 10.00 h from the jugular vein of fasting rats anaesthetised by sodium pentobarbital. The samples were taken into tubes without any anticoagulant. After the samples were allowed to stand at room temperature for 2 h, the serum was separated by centrifugation at 1500 *g* for 20 min. At the end of the 4-week experimental period, all faeces excreted during last 3 d were collected. The rats were anaesthetised with sodium pentobarbital and killed, and the livers and caecum were quickly removed, washed with cold saline (9g NaCl/L), blotted dry on filter paper, and weighed before freezing for storage. Liver aliquots for RNA isolation were stored at -80ºC; other samples were stored at -20ºC.

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. Preparation of potato peptides

The peptide preparations were done by an enzymatic hydrolysis method as described previously (Liyanage et al., 2008) and the preparation method of PPL was slightly different from PPS due to the long hydrolyzing period (25 h for PPL and 16 h for PPS) and higher recovery percentage (40% for PPL and 25% for PPS). Except the hydrolyzing period, all the other procedures were exactly same in both PPS and PPL preparations. The amino acid composition of PPL preparation was measured as previously described (Liyanage et al., 2008), which was not different from PPS preparation (Table 2). Amino acid sequences of 11 selected peptides in PPS mixture were determined by sequential Edman degradation on Procise 494HT Protein

Sequencing System using Pulse Liquid Program (Applied Bio System, Tokyo). The molecular weight of the PPL preparation was determined as described previously (Liyanage et al., 2008) and that was similar to PPS preparation, in the range of from 700 to 1840 Da (Fig.1). The MALDI-ToF spectrum in Fig 1B(for PPL) clearly shows a much flatter base line compared to Fig 1A(for PPS), indicating that PPS is a much more complex mixture of peptides than PPL, even though the 850Da peak is predominant in both. However, using MALDI-ToF spectrum cannot detect components smaller than 500 daltons and, and also cannot be used for quantitative assessment of the relative proportions of the components of different size.

The compositions of PPS and PPL were as follows (as %): moisture, 2.9 and 3.9; protein, 78.7 and 75; lipid, 0.6 and 0; carbohydrate, 12.5 and 11.9; ash, 5.3 and 9.2. Total moisture, protein, lipid and carbohydrate contents were determined by the procedure of the Association of Official Analytical Chemists (AOAC, Arlington, 1999).

2.3. Chemical analysis

Total cholesterol (TC), HDL-cholesterol (HDL-C), and triglyceride (TG) concentrations in the serum were determined enzymically using commercially available reagent kits (assay kits for the TDX system; Abbott Laboratories Co., Irving, TX, USA). The non-HDL cholesterol concentration was calculated as follows: [non-HDL-C] = [TC]-[HDL-C].

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 total lipid obtained by saponification were acetylated (Matsubara et al,1990) and analyzed by GLC with a Shimadzu 14A chromatograph (Kyoto, Japan) fitted with a DB17 capillary column (0.25mm×30m; J&W Scientific, Inc., Folsom, CA, USA) with N_2 as the carrier gas. Acidic sterols in the faeces were measured by GLC according to the method of Grundy et al., 1965). A part of the caecum was taken out into desalting water in a vial with out 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 SCFA was measured by GLC with 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. Ribonucleic acid (RNA) isolation, reverse transcription-polymerase chain reaction (RT-PCR), and southern blot analysis

Total RNA was isolated from the liver by the acid guanidinium-phenol-choloroform method using Isogen (Nippon Gene, Tokyo, Japan) (Chomczynski and Sacchi, 1987). mRNA encoding scavenger receptor class B type 1(SR-B1), the LDL receptor (LDL-R), sterol-regulatory element-binding protein (SREBP)-2, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, cholesterol 7-α hydroxylase (CYP7A1), apolipoprotein B(apo B), fatty acid synthase (FAS), sterol-regulatory element-binding protein (SREBP)-1c and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; used as an invariable control) were analyzed by semi-quantitative RT-PCR and subsequent southern hybridization of the PCR products with each inner oligonucleotide probe. Total RNA samples were treated with DNase RQ1 (Promega, Madison, WI, USA) to remove genomic DNA and subjected to RT-PCR by using Moloney murine leukemia virus RT (GIbco-BRL, Gaithersburg, MD, USA) and EX-Taq polymerase (Takara, Tokyo, Japan). Primers for

SR-B1, LDL-R.HMG-CoA reductase, CYP7A1, apo B, FAS, SREBP-1C and GAPDH were as described previously (Liyanage et al., 2008, Nakamura et al., 2009, Ruvini et al., 2007). The reaction mixture for PCR contained 25 pmol of each primer, 1.25 U of EX-Taq polymerase, 1 x PCR buffer (Takara), and 200 μM-dNTP in a 50 μL reaction volume. The initial temperature cycle was denaturation at 94°C for 3 min, annealing at 60°C for 1 min, and extension at 72°C for 2 min. Subsequent cycles were denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 2 min. The thermal cycle was completed by terminal extension at 72°C for 10 min. In total, 25 cycles were performed for LDL-R, SREBP-2, HMG-CoA R, SREBP-1c and GAPDH, 22 cycles for CYP7A1, apo B, and 20 cycles for FAS and SR-B1. The amplification products were electrophoresed on 2% agarose gel and transferred to a nylon membrane (Biodyne B; Pall Bio-Support, East Hills, NY, USA). The blots were hybridized with relevant probes as described previously (Liyanage et al., 2008, Nakamura et al., 2009, Ruvini et al., 2007).

The probe was 3′-tailing labeled with digoxigenin, using a DIG oligonucleotide tailing kit (Boehringer Mannheim, Mannheim, Germany). Prehybridization, hybridization, and detection were carried out with a DIG luminescent detection kit (Boehringer Mannheim) as recommended by the manufacturer. The relative quantity of mRNA was estimated by densitometric scanning with X-ray film.

2.5. Statistical analysis

Data are presented as the mean and standard deviation for serum TC, HDL-C, non-HDL-C, and TG level at the prescribed times. The significance of differences among treated groups was determined by analysis of variance and Duncan's multiple range test (SAS Institute, Cary, NC, USA). Differences were considered significant at *P*<0.05.

3. Results

3.1. Bodyweight, food intake, feed efficiency, liver, caecal, and faecal weight

There was no difference in the body weight, feed efficiency, and liver weight among the groups. But the food intake (g) was lower in PPS- (386.7±17.6[b]) fed group relative to CN- (449.2±21.1[a]) and PPL- (431.1±38.6[a]) fed groups. Faecal weight (g) was higher in both PPS- (1.14±0.12[a]) and PPL- (1.29±0.18[a]) fed groups relative to CN- (0.92±0.17[b]) fed group. Caecal weight (g) was higher in PPS- (4.87±0.90[a]) fed group compared with the PPL-(3.41±0.81[b]) fed group and not different compared with the CN-(3.90±0.53[ab]) fed group.

3.2. Serum lipid levels

The serum TC and serum TG concentration were lower in PPS-fed group compared with the CN-and PPL-fed groups, at the end of the 4-week feeding period. Serum non-HDL-C concentration was lower in both PPS-and PPL-fed groups relative to CN-fed group, and that in PPS-fed group was lower than the PPL-fed group, at the end of the 4-week feeding period. There was no difference in the serum HDL-C concentration among groups (Table 3).

3.3. Liver cholesterol, faecal lipids, caecal pH, and caecal lipids

Liver cholesterol level was higher in both PPS-and PPL- fed groups relative to

CN-fed group (Table 4). Faecal total lipid concentration was higher in PPS-fed group compared with the CN-and PPL-fed groups. Faecal cholesterol level was higher in PPL-fed group than the CN-fed group. Faecal coprostanol and neutral sterol concentrations were higher in both PPS-and PPL-fed groups than the CN-fed group. Faecal acid sterol concentration in PPL-fed group was lower, and that in PPS-fed group was not different, compared with the CN-fed group. Faecal cholic acid concentration was lower in both PPS-and PPL-fed groups compared with CN-fed group. Moreover, faecal chenodeoxycholic, and deoxycholic acid concentrations were lower in PPL-fed group, relative to CN-fed group. Faecal lithocholic acid concentration was lower in PPL-fed group relative to PPS-fed group. Faecal total bile acid concentration was lower in PPL-fed group than the PPS- and CN-fed groups (Table 4).

Caecal pH was not different among groups (data not shown). Caecal acetic acid, propionic acid, butyric acid and total SCFA concentrations were higher in PPL-fed group than those in the CN-and PPS-fed groups (Table 4).

3.4. Hepatic mRNAs

The relative quantities of mRNA were determined by the southern hybridization of PCR-amplified SR-B1 cDNA, LDL-R cDNA, SREBP-2 cDNA, HMG-CoA reductase cDNA, CYP7A1 cDNA, apo B cDNA , FAS cDNA and SREBP-1c cDNA in the rat liver. The values of SR-B1, LDL-R, SREBP-2, HMG-CoA R, CYP7A1, apo B, FAS and SREBP-1c mRNAs were normalized to the value of GAPDH mRNA. Values from liver samples from rats fed with PPS and PPL were expressed relative to the average values of control group, which were normalized to 100. There was no difference in relative quantity of CYP7A1 (Fig.2C), apo B, FAS and SREBP-1c mRNA level (data not shown) among groups. The LDL-R (Fig. 2A) and SR-B1 (Fig. 2B) mRNA level were higher in both PPS and PPL-fed groups compared with CN- fed group. The SREBP-2 (Fig. 2D) and HMG-CoA R (Fig. 2E) mRNA level were higher in PPL-fed group compared with CN-fed groups.

4. Discussion

In the present study we compared the effects of two different potato peptide preparations (PPS, PPL) on serum lipids, liver cholesterol, faecal lipids, caecal lipids, and hepatic mRNA in rats fed on a cholesterol-free diet in comparison with casein (CN). According to the results, there was no difference in the body weight gain, feed efficiency, liver weight, in peptide diet-fed groups relative to CN-fed group. However, the food intake was lower in PPS-fed group compared with other two groups, which was in line with our previous study (Liyanage et al., 2008), suggesting that some peptide fragments in PPS mixture may have suppressed the food intake, and PPL mixture may be free from those peptides or peptide fragments. The serum TC, non-HDL-C and TG concentrations in PPS-fed group were lower compared with the other two groups, suggesting that PPS preparation has a higher hypocholesterolaemic and hypotriglycerolaemic ability than the PPL preparation. Lower non-HDL-C level in both PPS-and PPL-fed groups was accompanied by higher LDL-R and SR-B1 mRNA levels, which are responsible for hepatic clearance of plasma lipoproteins (Gouni-Berthold and Sachinidis, 2004; Han et al., 2004). Moreover, we found that PPL up regulated the genes involved in cholesterol synthesis and cholesterol uptake *via* a increased level of mRNA coding for SREBP-2. Hepatic SREBP-2 mRNA level in

PPL- and CN-fed groups was positively correlated with HMG-CoA R and LDL-R mRNA level, correlation coefficients being, ($r = 0.837$, $P<0.01$), and ($r = 0.642$, $P<0.05$) respectively. SREBP-2 has been identified as a transcription factor responsible for the transcription activation of HMG-CoA R and LDL-R (Horton et al., 2002; Vallet et al., 1996). However, those hepatic genes in PPS-fed group were not modulated with the diet, suggesting that smaller or peptides with some specific fragments in PPL preparation might have easily penetrated through cell membranes and modulated some hepatic genes related to lipid metabolism and maintained the sterol balance. It was suggested that smaller peptides have the ability to penetrate plasma cell membrane and prevent oxidative cell death (Szeto, 2006).

The lower TC, non-HDL-C, and TG levels in PPS-fed group were followed by higher faecal total lipid, coprostanol and faecal neutral sterol level compared with the CN-and PPL-fed group. This suggests that PPS preparation may have a higher sterol binding capacity or micelle forming ability than the PPL preparation and promote faecal sterol excretion as reported previously for other dietary peptides (Nagaoka et al., 1999; Nagaoka et al., 2001). The faecal acidic sterol level was lower in PPL-fed group, and that in PPS-fed group was not different relative to CN-fed group, and in agreement with previous findings saying that low molecular weight peptides derived from food proteins lowered the serum cholesterol without excretion of cholesterol and bile acid (Yoshikawa et al., 2000).

On the other hand, caecal total SCFA, acetate, propionate, and butyrate concentration were higher in PPL-fed group compared with CN-and PPS-fed group, suggesting that PPL peptide mixture might have proliferated the caecal fermentation. However, the PPS preparation increased the caecal SCFA concentration when it was enriched with cholesterol in our previous study (Liyanage et al., 2009). Higher SCFA concentration in PPL-fed group may at least be partially responsible for lower non-HDL-C level, *via* induced faecal sterol excretion as observed previously for resistant starch and dietary fibres (Han et al., 2003; Illman et al., 1993). In fact, the substratum difference between two peptide preparations may have altered the residence time and the fermentation ability of caecum. It has been shown that movement of digesta through the colon is stimulated by caecal butyrate concentration, there by promoting gastro intestinal transit time and normal laxation (Yajima, 1985). Higher SCFA concentration in the PPL-fed groups was further supported by higher neutral sterol excretion in PPL-fed group. Serum non-HDL-C level was negatively correlated with serum total short chain fatty acid concentration in the PPL-, and CN-fed groups, correlation coefficient being, $r = -0.763$, $P<0.01$. Liver cholesterol level in all 3 dietary groups was negatively correlated ($r=-0.749$, $P<0.01$) with serum non-HDL cholesterol level, giving evidences that higher liver cholesterol level in both peptide diet-fed groups, may be due to a compensatory response to lower non-HDL-C level in both peptide diet-fed groups. Lower non-HDL-C level in both peptide diet-fed groups may have suppressed the VLDL production in the liver, resulting higher liver cholesterol level.

Findings of our previous studies showed that PPS preparation and soy peptides reduced serum non-HDL-C level when rats were fed either on a cholesterol free diet or cholesterol enriched diet (Liyanage et al., 2008, Liyanage et al., 2009). Thus, potato peptides could be considered as functional candidates with promising hypocholesterolaemic ability comparable to soy peptides.

In conclusion, this study has demonstrated that both potato peptide mixtures have the ability to reduce serum non-HDL cholesterol level compared with the CN diet *via* increased neutral sterol excretion. According to the findings it could be speculated that sterol binding capacity of PPS was higher than the PPL diet leading to greater hypocholesterolaemic ability. Whereas, the PPL preparation is highly fermentable in the caecum, the theory that caecal SCFAs are involved in the hypocholesterolaemic action of PPL seems tenable. Because of obvious differences in serum cholesterol level, serum triglyceride level and sterol binding capacity, it is likely that PPS and PPL differ in their hypocholesterolaemic and hypotriglycerolaemic potential and structural and substratum differences between two preparations are responsible for these effects. However, the amino acid composition, and the main molecular weight of two potato peptide preparations were not different from each other, and the reason for different hypocholesterolaemic responses could be due to some other reasons such as specific peptide fragments, and number of free amino acids. The primary structure of protein that is amino acid sequence determines how the chain twists and turns, which determines how it interacts with other molecules, and the difference in the length of peptides, which is substratum difference, determines how the peptides bind with other molecules. From previous studies it has been shown that substratum and structural difference of peptides determine how peptides interact with other molecules (Wang et al., 1995; Cheng and Seetharama., 2007; Kaushal et al., 2009).

Thus, the difference in hydrolyzation in peptide preparation may have caused substratum and structural difference in two potato peptide mixtures, causing differences in lipid modulation ability. Despite the low recovery percentage, PPS preparation has shown a greater hypocholesterolaemic and hypotriglycerolaemic ability than the PPL preparation.

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Appendix

CN; casein, PPS; short hydrolysis preparation, PPL; long hydrolysis preparation ,CHO; cholesterol, COPRO; coprostanol, TC; total cholesterol, HDL-C; High density lipoprotein cholesterol, TG; triglycerides, TBA; total bile acids, CA; cholic acid, CDCA; chenodeoxycholic acid, DCA; deoxycholic acid, LCA; lithocholic acid, GAPDH; glyceraldehyde-3-phosphate dehydrogenase, RT-PCR; reverse transcriptase-polymerase chain reaction, SCFA; short-chain fatty acids, LDL-R; LDL receptor, SR-B1; scavenger receptor class B type 1, SREBP-2; sterol-regulatory-element binding protein-2, HMG-CoA R; 3-hydroxy-3-methylglutaryl coenzyme A reductase, CYP7A1; cholesterol 7α-hydroxylase, ANOVA; analysis of variance.

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Figure captions

Fig. 1A Intensities of PPS preparation analyzed by MALDI-TOF mass spectrum

Fig.1B. Intensities of PPL preparation analyzed by MALDI-TOF mass spectrum

Fig. 2A. Hepatic Low density lipoprotein receptor expression in rats fed potato peptide diet for 4 weeks. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CN, casein diet; PPS; short hydrolysis preparation; PPL; long hydrolysis preparation. Values are means for five rats, with standard deviations represented by vertical bars. [a,b]Mean values with unlike letters were significantly different (P<0.05).

Fig. 2B. Hepatic scavenger receptor class B type 1 expression in rats fed potato peptide diets for 4 weeks. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CN, casein diet; PPS, short hydrolysis preparation; PPL, long hydrolysis preparation. Values are means for five rats, with standard deviations represented by vertical bars. [a,b]Mean values with unlike letters were significantly different (P<0.05).

Fig. 2C. Hepatic cholesterol 7α-hydroxylase expression in rats fed potato peptide diets for 4 weeks. GAPDH, glyceraldehyde-3-phosphate dehydrogenase;

CN, casein diet; PPS, short hydrolysis preparation; PPL, long hydrolysis preparation. Values are means for five rats, with standard deviations represented by vertical bars. [a,b]Mean values with unlike letters were significantly different (P<0.05).

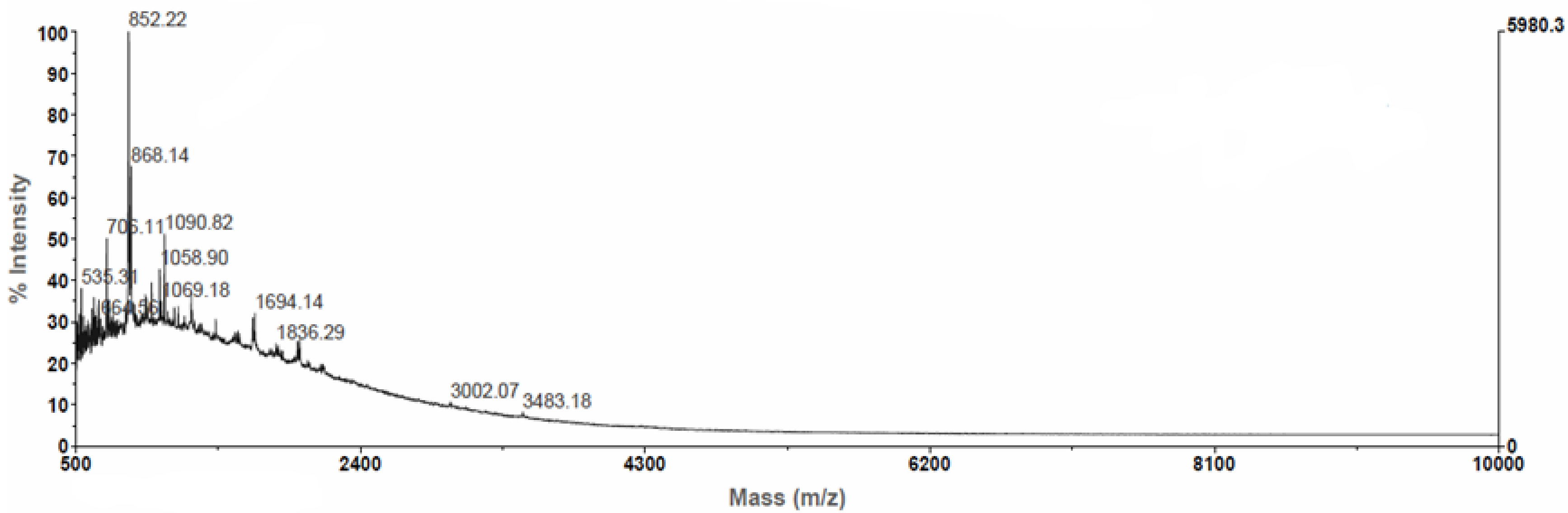
Fig. 2D. Hepatic sterol-regulatory element-binding protein (SREBP)-2 expression in rats fed potato peptide diets for 4 weeks. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CN, casein diet; PPS, short hydrolysis preparation; PPL, long hydrolysis preparation. Values are means for five rats, with standard deviations represented by vertical bars. [a,b]Mean values with unlike letters were significantly different (P<0.05).

Fig. 2E. Hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase expression in rats fed potato peptide diets for 4 weeks. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CN, casein diet; PPS, short hydrolysis preparation; PPL, long hydrolysis preparation. Values are means for five rats, with standard deviations represented by vertical bars. [a,b]Mean values with unlike letters were significantly different (P<0.05).

Table 1. Amino acid compositions of potato peptide preparations

Amino acids	CN	PPS (g/100g)	PPL
Aspartic acid	6.22	8.94	10.40
Threonine	3.65	4.22	4.99
Serine	4.59	3.60	4.49
Glutamic acid	18.90	8.43	9.58
Glycine	1.62	3.52	4.03
Cysteine	0.43	0.17	0.62
Valine	5.94	4.12	5.08
Methionine	2.70	1.56	1.86
Isoleucine	4.86	3.78	4.09
Leucine	8.38	7.78	8.00
Tyrosine	5.00	3.38	4.11
Phenylalanine	4.59	3.96	4.25
Lysine	7.16	4.93	5.98
Histidine	2.70	1.27	1.59
Arginine	3.24	3.54	3.76
Alanine	2.70	3.96	4.53
Proline	10.10	3.54	3.81

A



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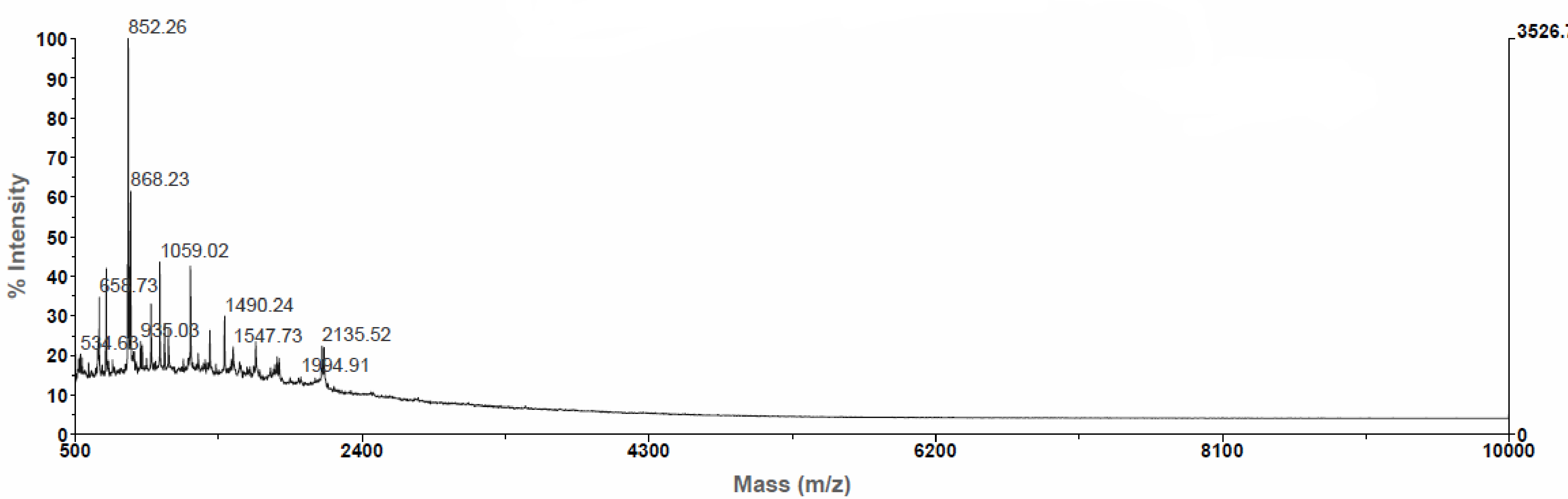

Fig. 1. Ruvini Liyanage

Table 2. Amino acid sequences of peptides in PPS preparation

Peak number	Sequence
1	Gly-Pro-His-Ile-Phe
2	Asp-Tyr-Phe-Asp-Val-Ile-Gly-Gly-Gly-Thr
3	Asp-Ile-Val-Pro-Phe
4	Asp-Tyr-Phe
5	Lys-Asp-Ile-Val-Pro-Phe
6	Glu-Ala-Ala-Lys-Trp-Gly-Pro
7	Ala-Ala-Lys-Trp-Gly-Pro
8	Ala-Lys-Trp-Gly-Pro-Leu
9	Tyr-Phe
10	Tyr-Phe
11	Phe-Asp-Lys-Thr-Tyr

Amino acid nomenclature: His, Histidine; Ile, Isoleucine;Val, Valine; Ala, Alanine; Gly, Glycine; Leu, Leucine; Pro, Proline; Thr, Threonine; Phe, Phenylalanine; Tyr, Tyrosine; Trp, Tryptophan; Asp, Aspartic acid; Glu, Glutamic acid; Lys, Lysine.

Table 3. Serum total cholesterol, HDL-cholesterol, non-HDL-cholesterol, and triglyceride concentrations in rats fed experimental diets for 4 weeks (mmol/l)

	Week 0	Week 2	Week 4
Total cholesterol			
CN	1.64 ±0.25	1.79 ±0.12	1.99 ±0.04[a]
PPS	1.54 ±0.06	1.64 ±0.09	1.72 ±0.06[b]
PPL	1.56 ±0.09	1.79 ±0.28	1.88 ±0.10[a]
HDL cholesterol			
CN	0.71 ±0.06	0.77 ±0.04	0.80 ±0.04
PPS	0.71 ±0.05	0.87 ±0.04	0.85 ±0.05
PPL	0.70 ±0.03	0.86 ±0.12	0.82 ±±0.06
Non-HDL- cholesterol			
CN	0.93 ±0.19	1.02 ±0.09[a]	1.18 ±0.04[a]
PPS	0.83 ±0.02	0.76 ±0.07[b]	0.87 ±0.06[c]
PPL	0.86 ±0.09	1.02 ±0.23[a]	1.06 ±0.07[b]
Triglyceride			
CN	0.64 ±0.25	1.49 ±0.17[a]	1.30 ±0.16[a]
PPS	0.46 ±0.23	0.90 ±0.09[b]	0.67 ±0.28[b]
PPL	0.44 ±0.21	1.27 ±0.39[a]	1.06 ±0.58[a]

CN, casein PPS; short hydrolysis preparation, PPL; long hydrolysis preparation.

[a,b,c]Mean values within a column with unlike superscript letters were significantly different (*P*<0.05).

Table 4. Liver cholesterol, faecal total lipids , neutral and acidic steroid excretion, and caecal short chain fatty acid concentrations in rats fed experimental diets for 4 weeks

		Dietary group		
		CN	PPS	PPL
Liver	CHO (mmol/g wet liver)	4.36 ± 1.27[b]	9.53 ± 3.25[a]	8.16 ± 1.97[a]
Faecal	T-Lipid (mg/g wet faeces)	36.2 ± 9.2[b]	83.5 ± 14.5[a]	51.4 ± 11.3[b]
Faecal	CHO (mmol/g wet faeces)	3.0 ± 2.1[b]	9.9 ± 3.5[ab]	16.2 ± 8.6[a]
	COPRO (mmol/g wet faeces)	3.1 ± 4.4[c]	30.8 ± 3.3[a]	15.0 ± 10.1[b]
Faecal	Neutral Sterol (mmol/g wet faeces)	6.1 ± 5.5[c]	40.7 ± 5.9[a]	31.1 ± 9.5[b]
	CA (mmol/g wet faeces)	0.68 ± 0.36[a]	0.27 ± 0.07[b]	0.22 ± 0.09[b]
	CDCA (mmol/g wet faeces)	0.43 ± 0.30[a]	0.23 ± 0.21[ab]	0.09 ± 0.06[b]
	DCA (mmol/g wet faeces)	0.22 ± 0.16[a]	0.13 ± 0.12[ab]	0.06 ± 0.03[b]
	LCA (mmol/g wet faeces)	0.53 ± 0.26[ab]	0.68 ± 0.41[a]	0.24 ± 0.16[b]
	TBA (mmol/g wet faeces)	1.87 ± 1.05[a]	1.30 ± 0.56[a]	0.61 ± 0.23[b]
Caecal	Acetic acid (μmol/g content)	49.2 ± 7.6[b]	35.7 ± 11.2[b]	74.3 ± 24.4[a]
	Propionic acid (μmol/g content)	7.0 ± 1.4[b]	4.9 ± 2.2[b]	12.1 ± 2.7[a]
	Butyric acid (μmol/g content)	5.8 ± 2.5[b]	5.3 ± 2.7[b]	11.6 ± 5.7[a]
	Total SCFA(μmol/g content)	62.0 ± 7.9[b]	45.9 ± 15.7[b]	98.0 ± 29.7[a]

CN, casein diet; PPS, short hydrolysis preparation; PPL, long hydrolysis preparation; CHO,cholesterol; T-Lipid,total lipid; COPRO,coprostanol;CA,cholic acid; CDCA,chenodeoxycholic acid; DCA,deoxycholic acid; LCA,lithocholic acid; TBA,total bile acids; SCFA, short chain fatty acids.

[a,b,c]Mean values within a row with unlike superscript letters were significantly different (P<0.05).

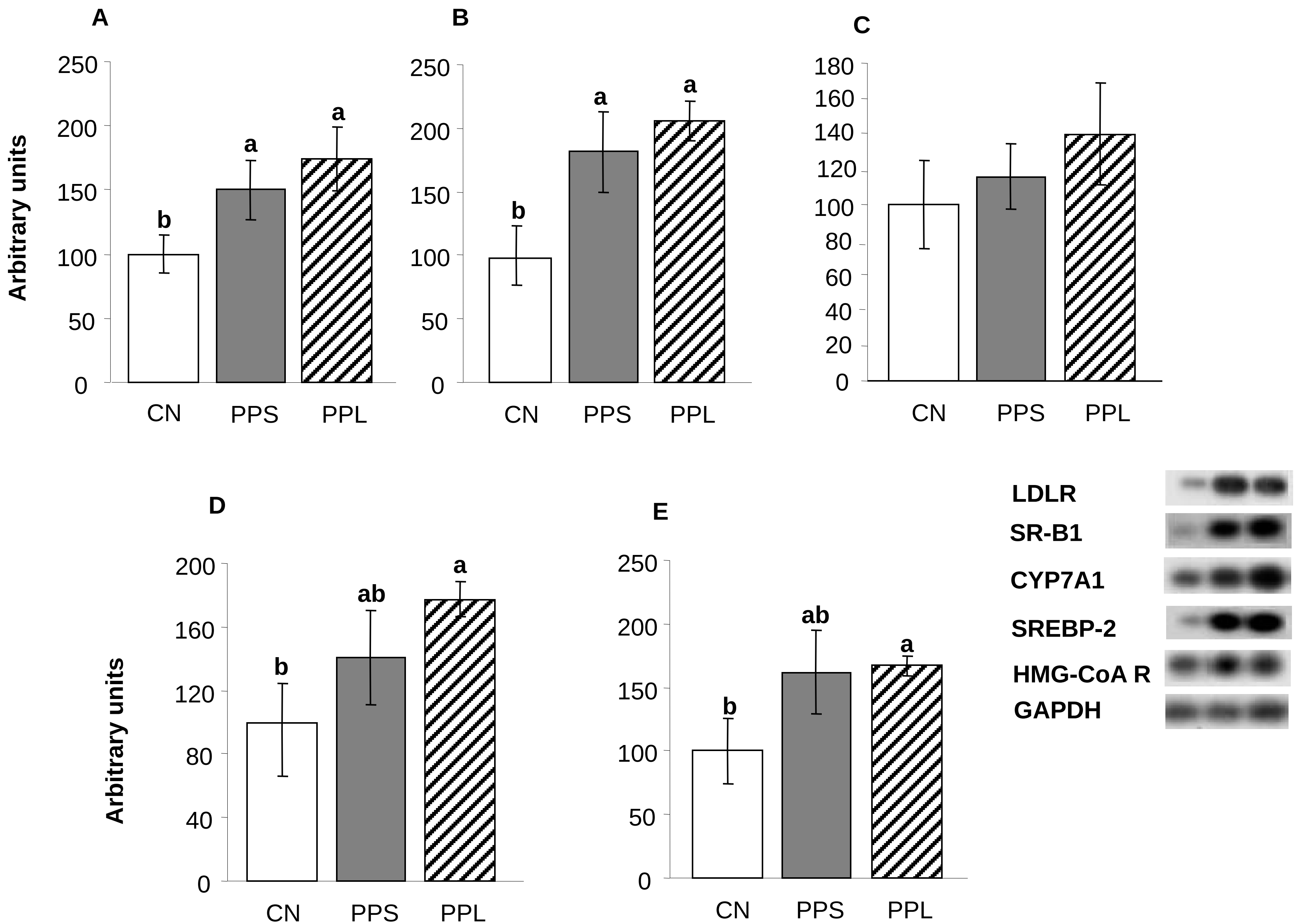

Fig. 2. Ruvini Liyanage