

## Amaranth protein presents cholesterol-lowering effect

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### ABSTRACT

This study describes amaranth's protein cholesterol-lowering effect and investigates its mechanisms hypercholesterolaemia was induced in male hamsters through diet rich in casein (300 g/kg diet) containing regular levels of cholesterol (0.5 kg/g) fed during 3 weeks. Animals were divided into three groups and fed *ad libitum* diets for 4 weeks containing as the sole source of protein: casein (control), amaranth protein isolate or, casein + amaranth protein isolate. Plasma concentrations of cholesterol and triacylglycerols were measured at four different points: at the beginning of the study, after hypercholesterolaemia was induced, in the first week and then at the end of the experimental diet period. The reduction of the total plasma cholesterol concentration at the end of experimental period for animals fed on diets containing amaranth protein isolate pure and with casein were 27% ( $P < 0.05$ ) and 48% ( $P < 0.05$ ), respectively, being the non-HDL fractions the most affected. Digestibility of protein as well as excretion of cholesterol and bile acid, were investigated as the possible mechanisms for this significant hypocholesterolaemic effect. Cholesterol excretion was related to the hypocholesterolaemia but could not explain all the observed reduction. Our findings suggest that amaranth protein has a metabolic effect on endogenous cholesterol metabolism.

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### 1. Introduction

Amaranth is a pseudo-cereal that has been widely grown by the Aztecs, Incas and Mayas in Latin America since pre-Columbian times for millennia. The small seeds contain considerable levels of high-quality protein (rich in lysine) and low levels of saturated fatty acids in their oil moiety, thus winning room on the health-food store shelves. In addition to its high nutritional value, amaranth grain has a hypocholesterolaemic potential (Berger et al., 2003; Chatuverdi, Sarojini, & Devi, 1993; Ferreira, Matias, & Arêas, 2007; Plate & Arêas, 2002). Several studies suggested that amaranth's oil fraction because of the favourable fatty acid profile and high content of some unsaponifiable components, as squalene, phytosterols, tocopherols and tocotrienols could be responsible for its beneficial hypocholesterolaemic effects. However, recent reports show that amaranth free of the lipid moiety is more efficient to produce reduction of blood cholesterol levels and conclude that other components, possibly the protein fraction, are responsible for these benefits (Berger et al., 2003; Plate & Arêas, 2002).

A wealth of evidence exists describing the beneficial effect of several grains on serum lipids soy bean (Carrol & Kurowska, 1995), sesame (*Sesamum indicum*) (Rajamohan & Kurup, 1997)

buckwheat (*Fagopyrum esculentum*) (Tomotake et al., 2001), lupine (*Lupinus albus* L.) (Sirtori et al., 2004), amaranth (*Amaranthus caudatus* L.) (Plate & Arêas, 2002), quinoa (*Chenopodium quinoa* Wild L.) (Takao et al., 2005) and cowpea (Frota, Mendonça, Saldiva, Cruz, & Arêas, 2008), have been reported to exhibit cholesterol-lowering effects. A number of different mechanisms may explain the favourable effects of these grains on cholesterolemia. A reduction in the intestinal absorption of cholesterol and/or bile acids, an increase in plasma cholesterol clearance through enhanced hepatic LDL-receptor activity, and changes in the hepatic biotransformation of cholesterol can all be implicated.

The present study was designed to evaluate the hypocholesterolaemic effect of amaranth protein in hypercholesterolaemic hamsters. Some mechanisms (higher rates of excretion of undigested protein, bile acids and cholesterol) underlying this effect were also investigated.

### 2. Materials and methods

#### 2.1. Protein isolate preparation

An amaranth protein isolate (API) was prepared from hexane-defatted amaranth (*Amaranthus cruentus*) flour as described by Arêas (1985) with protein extraction at pH 11 and subsequent isoelectric precipitation at pH 5.7.

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Aqueous dispersion of this flour (1:5, w/v) was brought to pH 11.0 with 1 M NaOH, and after mixing for 4 h at 25 °C, was then centrifuged at 9000×g for 20 min, at 4 °C. The supernatant fraction was adjusted to pH 5.7 with 1 M HCl and centrifuged again. Finally, the protein pellet was resuspended in water (1:5, w/v), adjusted to pH 7.0 with 1 M NaOH, and then lyophilised. The resulting powder was submitted to further defatting with ethanol to guarantee the absence of confounding hypocholesterolaemic compounds such as squalene.

The presence of squalene in amaranth protein isolate was determined in the fatty acid determination described below (Moreda, Perez-Camino, & Cert, 2001). Whole grain amaranth yielded approximately 6.1 g squalene/100 g of oil, whilst the protein isolate (prior to alcohol defatting) contained only 0.48 g squalene/100 g of oil. Following alcohol defatting, squalene was not detected.

## 2.2. Amaranth seed and protein isolate composition analysis

Water and ash contents were determined gravimetrically, whilst total protein by the Kjeldahl method, fat by diethyl ether extraction in a Soxhlet apparatus, crude fibre by the enzymatic-gravimetric method and carbohydrate content by difference (AOAC, 1993).

## 2.3. Amino acid analysis

To determine amino acid profile of amaranth whole grain and its protein isolate, the samples were hydrolysed with 6 M HCl. The separation and quantification of amino acids were then performed by ion exchange chromatography following post column derivatisation with nihydrine in an amino acid analyser (Dionex DX300) (Spackman, Stein, & Moore, 1958). Prior oxidation with performic acid was performed before 6 M HCl hydrolysis for proper determination of cysteine and methionine (Spindler, Stadler, & Tanner, 1984). Tryptophan was determined spectrophotometrically according to Spies (1967).

The amino acid profile of amaranth presented in Table 2 show an arginine/lysine ratio of 0.63 in whole grain samples, and of 0.51 in protein isolates. Casein used in the control diet presented the ratio of 2.50.

## 2.4. Animals, diets and feeding procedures

Golden Syrian hamsters, 21–26 days old and weighing 56 ± 13 g, were housed individually under a 12 h light-dark cycle in a temperature-controlled environment. After 5 days of adaptation time, seven animals were killed to determining the basal levels of blood lipids. The remaining ( $n = 35$ ) was fed *ad libitum* on a high casein (30%) diet (HYPER) for 3 weeks to induce hypercholesterolaemic state.

At the end of this period, blood was collected in 15 hamsters to ensure that hypercholesterolaemia was achieved. The animals were then randomly assigned to three distinct groups ( $n = 11$  or 12 per group) that differed only in protein composition: a control group (20% casein), amaranth group (20% amaranth protein isolate [API]) and casein + amaranth protein isolate group (20% casein + 10% API). Hamsters were fed on these purified diets *ad libitum* for four weeks. During the third week of experimental diet, animals were put in cages containing suspended stainless steel platform for faeces collection.

Diets were designed to provide similar amounts of proteins, carbohydrates, lipids, vitamins, minerals, cholesterol and cholic acid as the control diet. The composition of diets is given in Table 2. The CAS + API diet was used to evaluate the hypocholesterolaemic potential of amaranth protein even in the presence of a hypercho-

lesterolaemic protein source (casein). This diet contained 30% protein, but it was still isocaloric to all others. To control for possible fatty acid composition influence on the measured lipid-lowering effect, the proportions of polyunsaturated, monounsaturated and saturated fats were kept equal (1:1:1) in all diets by mixing coconut, canola and sunflower oils. The daily food consumption was recorded, and fresh food was provided every day. Body weights of hamsters were monitored on a weekly basis.

## 2.5. Fatty acid analyses

Fatty acid profiles of the diets were determined using dry-column lipid extraction method (Marmer & Maxwell, 1981), followed by lipid derivatisation to produce fatty acid methyl esters (Hartman & Lago, 1973). The fatty acids determination was carried out on Chrompack CP9200 Gas Chromatograph (GC). The GC was equipped with a CP-Sill 88 fused silica column (50 m, 0.25 mm I.D., film thickness 0.25 µm; Nordion, Helsinki, Finland). The oven temperature was programmed to rise from 100 to 235 °C at the rate of 10 °C/min under the following conditions: carrier gas H<sub>2</sub> (2.5 ml/min), split ratio 50:1, flame ionisation detector (FID) 250 °C. The peaks of fatty acids were identified by comparison of the retention time with FAME standard (Supelco, Code: 189-19, Sigma). The fatty acid profile of all diets did not differ significantly, containing 43.2–45% of saturated, 26–27.3% of monounsaturated and 28.1–30.9% of polyunsaturated fatty acids. The cholesterol content of the diets was determined after dry-column extraction (Marmer & Maxwell, 1981), saponification (Bohac, Rhee, Cross, & Ono, 1988) and quantification in a Shimadzu HPLC, equipped with diode array UV-vis detector, with Luna Phenomenex linked with ciano column (5 µm) 4.6 × 150 mm and mobile phase consisting of hexane: isopropanol (97:3 v/v) solution flowing at 1 ml/min. The run took 7 min and spectra and chromatograms were taken at 190–300 nm and 206 nm, respectively. Quantification was carried out by daily external standardisation using cholesterol standard (Sigma, St. Louis, USA) to build the reference curve. The peak's identity and purity were verified and corrected by means of spectra obtained with the photodiode array detector. Class-VP 10 was the software used (Shimadzu Co. Japan).

## 2.6. Blood and faecal analyses

Blood was drawn from seven randomly selected hamsters in the beginning of the study to establish their basal plasma lipid levels. Another draw was performed following 3 weeks of hypercholesterolaemic diet. Thereafter a draw was performed five days after the initiation of experimental diets ( $n = 5$  of each group) and another at the end of experimental period (4 weeks). In all instances blood draws were executed via cardiac puncture performed under anaesthesia with ketamine (85 mg/kg) and xylazine (8.3 mg/kg) after 15 h fast.

Blood samples were drawn into a heparin-wetted syringe, and plasma was harvested after centrifugation at 1500×g for 15 min. Plasma total cholesterol (TC) and triacylglycerol (TAG) concentrations were measured with commercial enzymatic assay kits (Labtest, Brazil). HDL-cholesterol was measured subsequent to the precipitation of the apo B-containing lipoproteins with sodium phosphotungstate magnesium chloride (Labtest, Brazil, catalogue number Cat 13). The supernatant fraction was assayed for total cholesterol using the enzymatic kit for total cholesterol (Weingand & Dagg, 1990). Cholesterol concentration in the VLDL + LDL fractions was expressed as non-HDL-cholesterol and calculated as the difference between total plasma cholesterol and HDL-cholesterol. Calculation of LDL-cholesterol using Friedewald's equation was inappropriate in this case due to the different distribution of lipids across lipoprotein groups in hamsters as compared to humans (Goulinet & Chapman, 1993).

Faecal samples were collected during the third week of the experimental diet. The animals were housed in wired-bottomed cages, and the faeces were collected every 24 h during 3 days. Faecal cholesterol concentrations were determined in the oven-dried faecal material following extraction with petroleum ether. The extract was dried, re-dissolved in 500–3000 µl of hexane:isopropanol (97:3) solution, filtered through a 0.45 µm membrane and injected in a HPLC system that was previously described.

Total faecal bile acid was measured in faeces extracts using diazyme enzymatic recycling rate assay kit (Cat. DZ042A). The oven-dried faeces were extracted with a mixture of *tert*-butanol:water (50:50) at 37 °C during 15 min. The samples were centrifuged at 10,000×g for 2 min (van der Meer, de Vries, & Glatz, 1985).

Animals were sacrificed under anaesthesia by hypovolemia at the end of experiment. The livers were washed with saline solution and weighted. The aortic arches were collected and kept in buffered formaldehyde until histological cuts and staining with hematoxyline–eosine were performed.

All experimental protocols and procedures were approved by Committee of Ethics in Research of Medical Faculty of São Paulo University (CAPesq number 871/04).

### 2.7. Statistical analysis

Values were presented as means with standard errors (±SEM). ANOVA test was used to analyse the significance of differences ( $P < 0.05$ ) amongst groups. Tukey's test was subsequently performed for mean comparisons, and Pearson coefficients were computed to determine the correlations between parameters. SPSS (Statistical Package for the Social Sciences) software for Windows 13.0 was used for the above analyses.

## 3. Results and discussion

Protein isolation was efficient and yielded an isolate with 96% of protein and minor amounts of other components (Table 1). Electrophoretic patterns of all stages of protein isolation (results not

**Table 1**  
Proximal and amino acid composition of *Amaranthus cruentus* whole grain and its protein isolate (g/100 g in dry base).

	Whole grain	Amaranth protein isolate (API)
Ash	2.1	1.2
Protein	13.9	96
Lipids	7.3	0.7
Dietary fibre	7.7	Na
Carbohydrates	69.0	2.1
Squalene (g/100 g of oil)	6.1	Nd
<i>Amino acid profile (g amino acid/100 g protein)</i>		
Asp	8.3	8.5
Thr	3.6	3.8
Ser	6.4	4.9
Glu	18.6	17.7
Pro	4.5	5.3
Gly	9.0	6.1
Ala	4.3	4.1
Cys	2.0	1.8
Val	4.4	5.1
Met	2.0	2.4
Ile	3.7	4.6
Leu	6.1	7.1
Tyr	3.3	3.9
Phe	4.6	5.1
Lys	6.2	5.3
His	2.6	2.9
Arg	9.8	10.4
Trp	2.2	1.8
Lys/arg ratio	0.63	0.51

Na – not analysed.

Nd – not detected.

**Table 2**

Composition of experimental diets (g/kg of diet).

Ingredients	Hyper	Control	CAS + API	API
Casein <sup>a</sup>	335	220	220	–
Amaranth protein isolate	–	–	1125	225
Mixture of oils <sup>b</sup>	150	150	150	150
Choline bitartrate	2	2	2	2
Cholesterol	0.5	0.5	0.5	0.5
Cellulose	50	50	50	50
Sucrose	150	150	150	150
Corn starch	267.5	377.5	382.5	270
Vitamin mix <sup>c</sup>	35	35	35	35
Mineral mix <sup>c</sup>	10	10	10	10
<i>Nutrient content (dry base)</i>				
Dry matter	938	935	954	895
Ashes	30	25	29	27
Crude protein ( $N \times 6.25$ )	319	185	297	205
Crude fat	158	162	152	153
Cholesterol	0.7	0.7	0.7	0.6
Carbohydrate <sup>d</sup>	493	628	522	615
Total energy (kCal/kg diet) <sup>e</sup>	4672	4710	4644	4657

<sup>a</sup> 93% of protein.

<sup>b</sup> 36% of sunflower oil + 28% of canola oil + 36% of coconut oil, generating the proportion of 1:1:1 of saturated/mono/polyunsaturated fatty acid profile of lipids.

<sup>c</sup> According to recommendations of AIN-93 for growing rodents (Reeves, Nielsen, & Fahey, 1993).

<sup>d</sup> Include dietary fibre.

<sup>e</sup> Protein  $\times$  4, carbohydrate  $\times$  4, lipid  $\times$  9.

shown) indicate minor changes in protein fractions. The gels were stained photographed and analysed by the software Gelworks 1D, Advanced (UVP, Cambridge, UK) presenting a similarity higher than 90% using dice comparison. Squalene, present in the whole seed at the concentration of 6.1/100 g was nearly completely removed by the isolation procedure to a concentration of 0.48/100 g of oil and was completely eliminated after ethanol washing of the isolate (Table 1). Ethanol washed amaranth protein isolate was used in the animal assays. Isocaloric diets were prepared according to the composition shown in Table 2. Digestibility of protein of all diets is shown in Table 3. It can be noticed that there were no significant differences amongst protein digestibility in all diets and that protein fed on the animals were completely digested.

As can be seen from Fig. 1 the amaranth protein alone was effective in significantly ( $P < 0.05$ ) lowering the plasma concentrations of total cholesterol, HDL-cholesterol, non-HDL-cholesterol and triacylglycerols. Previous experiments with amaranth showed that amaranth presents cholesterol lowering properties (Berger et al., 2003; Chatuverdi et al., 1993; Plate & Arêas, 2002). Plate and Arêas (2002), verified that the effect of amaranth oil in lowering total and LDL-cholesterol in hypercholesterolaemic rabbits was negligible. However, defatted and extruded amaranth promoted a remarkable reduction of hypercholesterolaemia. Berger et al. (2003) also found that plasma lipids were not significantly affected by any treatment

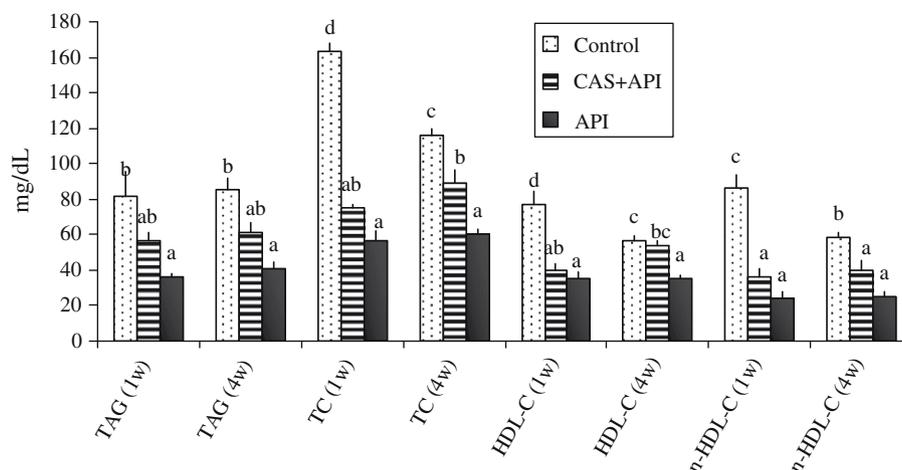
**Table 3**

Physiological data and relative organ weights in hamsters fed different experimental diets for 4 weeks (means  $\pm$  standard error).

	Control	CAS + API	API
Daily diet intake (g/day)	6.73 $\pm$ 0.19 <sup>b</sup>	5.89 $\pm$ 0.40 <sup>a,b</sup>	5.24 $\pm$ 0.32 <sup>a</sup>
Total diet intake (g)	150.9 $\pm$ 3.96 <sup>a</sup>	152.9 $\pm$ 9.64 <sup>a</sup>	134.1 $\pm$ 6.88 <sup>a</sup>
True digestibility (%)	101.0 $\pm$ 0.40 <sup>a</sup>	99.2 $\pm$ 0.38 <sup>a</sup>	100.6 $\pm$ 0.98 <sup>a</sup>
Liver g/100 g BW	4.24 $\pm$ 0.13 <sup>b</sup>	3.37 $\pm$ 0.13 <sup>a</sup>	3.08 $\pm$ 0.10 <sup>a</sup>

BW = body weight, control: casein diet; CAS + API: casein + amaranth protein isolate; API: amaranth protein isolate.

<sup>a,b,c</sup>Values sharing a common letter at the same line are not significantly different (Tukey test;  $P < 0.05$ ).



**Fig. 1.** Plasma lipid and lipoprotein cholesterol concentrations in hamsters fed different diets in two moments of experiment: first and fourth week of experimental diet. TAG: triacylglycerol, TC: total cholesterol, LDL-C: cholesterol of LDL, HDL-C: cholesterol of HDL, n-HDL: non-HDL-cholesterol. <sup>abc</sup>Values sharing a common letter for the same plasma parameter are not significantly different (Tukey test  $P < 0.05$ ).

with amaranth oil and unsaponifiable fraction but treatment containing amaranth protein decreased the total cholesterol in 10% after 28 days.

The reduction of total plasma cholesterol of the animals receiving diets with amaranth protein alone was 48%. This figure dropped to 27% when it was admixed with casein. For the non-HDL-cholesterol the decreases were 57% and 39%, respectively. All reductions were significant when compared to the casein control ( $P < 0.05$ ).

Our results show that the amaranth protein cholesterol lowering effect is not a consequence of removal of casein, a hypercholesterolaemic inducer, from diets. We found that even in the presence of the same amount of casein as in the control diet, the amaranth protein added in the diet still caused a significant reduction in total plasma cholesterol and non-HDL-cholesterol.

The observed decrease in total/HDL-cholesterol ratio indicates that amaranth protein may have a useful effect on lipid metabolism and can reduce cholesterol levels.

Some reports of protein consumption indicated total plasma cholesterol reduction with increased neutral sterol excretion (Frota et al., 2008; Tomotake et al., 2001). Protein's lower digestibility was suggested to have a dietary fibre-like mechanism. In the present study although we found also elevated faecal excretion of neutral sterols, this was neither associated with lower digestibility of the protein (Table 3) nor with increased bile acid output (Table 4). The total sterol excretion (the sum of cholesterol and bile acid excretion) increased significantly in the hamsters on amaranth protein containing diets.

Carr, Wood, Hassel, Bahl, and Gallaher (2003), investigating the effects of dietary fibre in hamsters, found that faecal cholesterol excretion is related to the hypocholesterolaemia, and that de-

creased bile acid excretion did not hinder cholesterol-lowering effect of dietary fibre.

Nagaoka and others (1999) reported that a peptic hydrolysate of soy protein had a cholesterol-lowering effect similar to that of intact soy protein. Not all soy protein is fully degraded by digestive enzymes and results in peptides that are absorbed as such. These enzyme-resistant soy peptides may have metabolic effects on cholesterol metabolism or also properties similar to those of dietary fibres in their ability to bind to bile acids and cholesterol and thus increase their faecal excretion, or both of these possibilities.

Liver weights calculated as percentages of body weight were higher in casein-fed hamsters than in hamsters fed amaranth protein containing diets (Table 3). The increased hepatic weight is most likely associated with lipid deposition. Although liver lipids were not determined, Pearson's correlation showed that the only independent variables that significantly correlated to liver weight was total plasma cholesterol (0.726,  $P = 0.016$ ) suggesting that its increase is related to protein interference on lipid metabolism resulting in lipid accumulation.

The observed cholesterol-lowering effects in the present study were mainly due to the reduction in non-HDL-cholesterol. It has been suggested that protein-induced alterations of cholesterol metabolism may be mediated by the differences in amino acid patterns of dietary proteins or by bioactive peptides (Yoshikawa, Takahashi, & Yang, 2003). The arginine/lysine ratio found in API was markedly lower (0.51) than in casein (2.20–2.30) and in soy protein isolate (0.80–0.90). However, this hypothesis has been dropped recently in favour of bioactive peptides produced by the incomplete digestion of some proteins. This is the case of soy protein where a series of peptides with distinct action on cholesterol synthesis and absorption and on LDL receptor synthesis have been

**Table 4**

Faecal cholesterol and total bile acids excretion in hamsters with different diets during the third week of experiment (means  $\pm$  standard error of mean).

	Hyper	Control	CAS + API	API
Weight of dried faeces (g/3 days)	1.305 <sup>ab</sup> ( $\pm 6 \times 10^{-2}$ )	1.702 <sup>b</sup> ( $\pm 6 \times 10^{-2}$ )	1.568 <sup>ab</sup> ( $\pm 8 \times 10^{-2}$ )	1.232 <sup>a</sup> ( $\pm 0.149$ )
Bile acid excretion ( $\mu\text{mol/d/BW}$ ) <sup>A</sup>	1.303 <sup>b</sup> ( $\pm 8 \times 10^{-2}$ )	1.326 <sup>b</sup> ( $\pm 6 \times 10^{-2}$ )	0.843 <sup>a</sup> ( $\pm 0.149$ )	0.593 <sup>a</sup> ( $\pm 0.107$ )
Cholesterol excretion ( $\mu\text{mol/d/BW}$ ) <sup>A</sup>	5.693 <sup>a</sup> ( $\pm 0.912$ )	10.868 <sup>ab</sup> ( $\pm 1.613$ )	20.880 <sup>bc</sup> ( $\pm 4.086$ )	26.617 <sup>c</sup> ( $\pm 4.609$ )
Total sterol excretion ( $\mu\text{mol/d/BW}$ ) <sup>A</sup>	6.996 <sup>a</sup> ( $\pm 0.912$ )	12.193 <sup>ab</sup> ( $\pm 1.613$ )	21.72 <sup>bc</sup> ( $\pm 4.153$ )	27.21 <sup>c</sup> ( $\pm 4.647$ )

<sup>abc</sup>Values sharing a common letter at the same line are not significantly different (Tukey test;  $P < 0.05$ ).

<sup>A</sup> Micromoles excreted in faeces per day per 100 g of animal body weight.

reported (Asato, Kina, Sugiyama, Shimabukuro, & Yamamoto, 1994; Cho, Juillerat, & Lee, 2008; Nagaoka et al., 1999; Wang & Ng, 1999).

Amaranth protein, whether used as replacement or supplementation of dietary protein showed an important hypocholesterolaemic effect in hamsters. The mechanism for this effect do not include dietary fibre-like action as the digestibility of amaranth protein was high and its effects on bile acid excretion were found to be unrelated. Amaranth protein intake led to remarkable decrease in non-HDL-cholesterol probably by reducing its synthesis. Bioactive peptides are likely to be produced by incomplete protein digestion and may affect cholesterol absorption in the gut or even their direct effect on cholesterol synthesis and/or LDL-receptor activity. Further studies are being conducted in our laboratories to clarify the mechanisms of the hypocholesterolaemic effect of this protein.

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