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The Vicilin protein (Vigna radiata L.) of mung bean as a functional food: Evidence of “in vitro” hypocholesterolemic activity

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The Vicilin protein (*Vigna radiata L.*) of mung bean as a functional food

Evidence of "in vitro" hypocholesterolemic activity

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Abstract

Purpose – The hypocholesterolemic activity of legume vicilins and the structural homology among mung bean, soybean and adzuki bean vicilins (8S) suggest that this protein may play a role in lipid metabolism. Thus, in the present study, the authors aim to isolate the mung bean vicilin and assess its in vitro effect on 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG CoAr), the enzyme responsible for endogenous cholesterol synthesis.

Design/methodology/approach – Chromatographic and electrophoretic characterization identified the molecular mass and polypeptide composition of mung bean vicilin. The hydrolysate of this globulin was obtained by sequential hydrolysis with pepsin-pancreatin and the fragments were characterized by molecular filtration, SDS PAGE and HPLC.

Findings – The molecular mass of vicilin was estimated as 158.23 at ± 10 kDa and SDS-PAGE revealed that the 8S globulin protein comprises four bands corresponding to polypeptides of 61, 48, 29 and 26 kDa. Fractions 10, 12, 14, 22 and 32 of the eluate from Sephadex G-25 exhibited significant inhibition of HMG CoAr.

Originality/value – The correspondence of the chromatographic profile of the peptide fractions with hypocholesterolemic activity suggests that the composition and chemical structure of these peptides are essential to their physiological effectiveness. The beneficial effects of mung bean vicilin identified in this study will support the characterization of this protein as a functional compound.

Keywords 8S globulin, Functional compound, HMG CoAr, Hypocholesterolemic activity, Mung bean

Paper type Research paper

1. Introduction

Mung beans originated in India and approximately 90 per cent of current mung bean production occurs in South, East and Southeast Asia. The medicinal properties of this plant

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have been widely reported for centuries, particularly in China, where the use of mung bean is quite common (Tang *et al.*, 2014; Nair *et al.*, 2013). The phenolic compounds, oligosaccharides and proteins present in mung bean exhibit antihypertensive, antitumor, hypolipidemic, antibacterial, antioxidant, anti-diabetic and anti-inflammatory activities (Tang *et al.*, 2014).

The protein content of mung bean (*Vigna radiata L*). ranges from 17 to 26 per cent (Mendoza *et al.*, 2001), comprising 62 globulins, 16.3 albumins, 13.3 glutelins and 0.9 per cent prolamins (Tsou *et al.*, 1979). Vicilin or 8S corresponds to 89 per cent of the total globulins followed by 7.6 legumin (11S) and 3.4 per cent basic 7S (Mendoza *et al.*, 2001). In addition to their nutritional importance, the physicochemical characteristics of green mung bean proteins appear to influence the functional properties of this legume and this applications in the food industry (Akaerue and Onwuka, 2010; Tang and Sun, 2010; Dzudie and Hardy, 1996). The physiological effects of mung bean proteins have also received much research attention. Yao *et al.* (2014) and Tachibana *et al.* (2013) identified *in vivo* hypolipidemic action of proteins isolated from mung beans, and Viernes *et al.* (2012) reported antihypertensive activity of 8S peptides derived from mung bean via inhibition of angiotensin converting enzyme activity.

The hypolipidemic action of legume proteins, particularly vicilins and legumins has been widely reported (Ferreira *et al.*, 2015; Spielmann *et al.*, 2008; Mayilvaganan *et al.*, 2004), and although the mechanisms underlying these not been fully elucidated, the structural homology between these species has been implicated (Tang and Sun, 2011; Mendoza *et al.*, 2001; Doyle *et al.*, 1985; Lackey, 1981). Thus, within the current perspective of research on functional compounds present in foods, the aim of to present study was to characterize the vicilin fraction and identify its hypocholesterolemic activity using *in vitro* assays.

2. Material and methods

2.1 Flour preparation from mung bean seeds

Mung beans seeds were obtained from the Empresa de Pesquisa Agropecuária de Minas Gerais at the Federal University of Viçosa, Minas Gerais, Brazil. Whole seeds were select, cleaned and macerated in distilled water at 4°C for 12 h. The shells were subsequently removed manually, and the cotyledons were dried in an oven at 45°C with air circulation. The dried beans were milled in a 60-mesh grain sieve, and the protein composition of the resultant flour was analyzed in accordance with the parameters of the AOAC (2010). The flour was also used to extract the vicilin fraction.

2.2 Isolation and characterization of the 8S globulin of mung bean

The 8S globulin of mung bean was extracted according to Sun and Hall (1975) with minor modifications. The mung bean flour was homogenized in 0.5 M sodium chloride buffer containing 0.025 M hydrochloric acid, 0.1 mM sodium fluoride and 5 mM β-mercaptoethanol, pH 3.5, at a ratio 1:20 (weight/volume) and kept under gentle shaking for 2 h at 4°C. This material was subsequently centrifuged at 30,000 g for 20 min at 4°C, and the supernatant was diluted in water at pH 3.0 at a ratio of 1:7 (volume/volume). After for 2 h the solution was decanted, and the decanted volume was centrifuged at 30,000 g for 20 min at 4°C. The resultant precipitate was washed twice with 0.080 M sodium chloride buffer, pH 4.5. The final precipitated pellet corresponded to the 8S globulin of mung bean.

The 8S globulin of mung bean was analyzed by elution on a column Sepharose CL-6B (2.5 $\times$ 100 cm) previously equilibrated with 0.01 M potassium phosphate buffer, pH 7.5, containing 0.5 M sodium chloride and 0.01 per cent sodium azide. The eluate was collected in 5.5 ml fractions and the absorbance of each fraction was read at 280 nm. The

chromatographic peak corresponding to 8S globulin was collected, stored and subjected to electrophoretic analysis and enzymatic hydrolysis *in vitro*.

An aliquot of the isolated 8S globulin fraction was also applied a Sephadex G-200 column (100 × 1.7 cm) previously equilibrated with 20 mM potassium phosphate buffer containing 0.5 M sodium chloride, pH 7.5. The absorbance of the eluated fractions was monitored at 280 nm absorbance. Cytochrome C (12.4 kDa), ovalbumin (45 kDa), bovine serum albumin (66 kDa), myosin (240 kDa) and ferritin (440 kDa) proteins were used as molecular mass standards. The chromatographic profile obtained from the elution of the globulin was used for electrophoretic analysis and molecular mass estimation.

The molecular masses of the 8S subunits were determined by gel electrophoresis on 12 per cent polyacrylamide gels in the presence of the reducing agent 2-mercaptoethanol according to Laemmli (1970). The know molecular masses of phosphorylase b (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa) were used as standards.

2.3 Preparation and characterization of the mung bean 8S globulin hydrolysates

The chromatographic peak 8S fraction obtained from the Sepharose CL 6B column was sequentially hydrolyzed with pepsin in potassium chloride-hydrochloric acid buffer, pH 1 and with pancreatin in 0.1 and 0.2 M sodium phosphate buffer, pH 8, with constant agitation at 37°C for different times (0 to 360 min). The enzymes were inactivated by altering the temperature (90°C for 20 min), according to Akeson and Stahmann (1964). To determinate of the degree of hydrolysis, the samples were analyzed according to the methodology of Fields (1972) with modifications according to Spadaro *et al.* (1979).

The peptide profile produced by hydrolysis pepsin-pancreatin was determined by SDS-PAGE Tricine, according to Haider *et al.* (2012). A molecular mass standard peptide marker kit (Sigma C-6210) containing peptides in the range of 1.06-26.6 kDa was used. The chromatographic profile of the hydrolysate was determined after elution on a Sephadex G-25 column, and the collected fractions were spectrophotometrically analyzed at 280 nm. The buffer was 5 mM potassium phosphate buffer containing 0.5 M sodium chloride and 0.01 per cent sodium azide, pH 7.5, a flow rate of 1.5 ml/3 min.

2.4 Quantification of the inhibition of HMG-CoA reductase activity and chromatographic profiling using high-performance liquid chromatography

The hypocholesterolemic activity of the peptide fractions eluted from a Sephadex G-25 filtration column was characterized as a percentage of the inhibition of the 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG CoAr) enzyme using a commercial HMG CoAr Assay kit (Sigma®) and pravastatina as a positive control. The assay is based on spectrophotometric analysis at 340nm in which the reduction of absorbance represents the oxidation of NADPH by the catalytic subunit of HMG-CoA, on the presence of HMG-CoA substrate, thus demonstrating inhibition of the enzyme activity. The statistical analyses were performed using the SigmaStat® 3.5 program (Dundas Software). Significant differences among the peptides fractions were determined by one-way ANOVA and Bonferroni t test multiple-range comparisons versus the positive control, with $p < 0.05$ as the criterion of significance.

The chromatographic profile of the peptide fractions with hypocholesterolemic activity was characterized by high-performance liquid chromatography (HPLC) on a Shimadzu system with an analytical C18 reverse phase column (0.46 × 25 cm; Kromasil). The chromatographic profile of the peptides was determined Solvent A (0.045 per cent TFA in

deionized water) and Solvent B (0.036 per cent TFA in acetonitrile) under the following conditions: automatic sample injection, 1 ml/min flow and 5-95 per cent B gradient in 30 min.

3. Results and discussion

Legume grains possess 20 to 40 per cent proteins, and globulins constitute 35 to 70 per cent of this total, with molecular masses between 140 and 200 kDa (Chel-Guerrero *et al.*, 2007; Duranti, 2006). In the present study, mung bean protein corresponded to 25.26 per cent of the nutrient content of the grain, and 65.03 per cent of protein was total globulins.

The extraction method provided a 96 per cent pure vicilin fraction according to the chromatographic profile obtained after elution on a Sepharose CL-6B column [Figure 1 (a)]. The molecular mass of vicilin was estimated as 158.23 at ± 10 kDa. SDS-PAGE (Figure 1 (b)) revealed that the 8S globulin protein comprises four bands corresponding to polypeptides of 61, 48, 29 and 26 kDa, consistent with Mendoza *et al.* (2001), Tang and Sun (2010) and Viernes *et al.* (2012).

The chromatographic peak from the Sepharose CL 6B column was subjected to sequential hydrolysis with pepsin and pancreatin proteolytic enzymes. In the presence of pepsin, a slight increase in the degree of hydrolysis was observed, with a considerable increase after the addition of pancreatin, yielding a 5 per cent degree of hydrolysis after digestion for 6 hours [Figure 2 (a)]. Rui *et al.* (2012) sequentially hydrolyzed nine varieties of beans (*Phaseolus vulgaris*) using trypsin, α-amylase, pepsin, trypsin and α-chymotrypsin and observed that the degree of hydrolysis of the obtained hydrolysates ranged from 1.72 to 16.12 per cent depending on the combination of enzymes and the variety of bean used. Rui *et al.* suggested that these beans were resistant to digestion and exhibited a low degree of hydrolysis. Moreover, the peptides obtained by Rui *et al.* exhibited antihypertensive activity, suggesting that bioactivity is associated with not only the degree of hydrolysis but also other factors, such as the hydrolysis method used, the protein composition and amino acid sequence. Analysis of the total hydrolysate of vicilin mung beans obtained from digestion

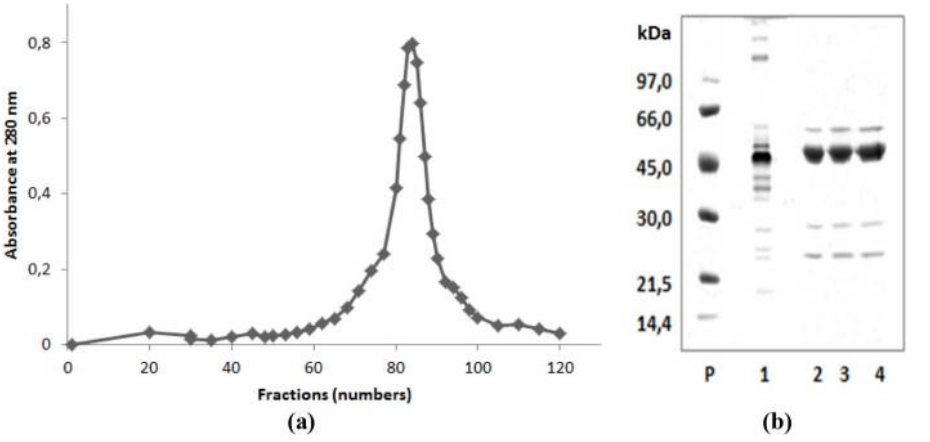


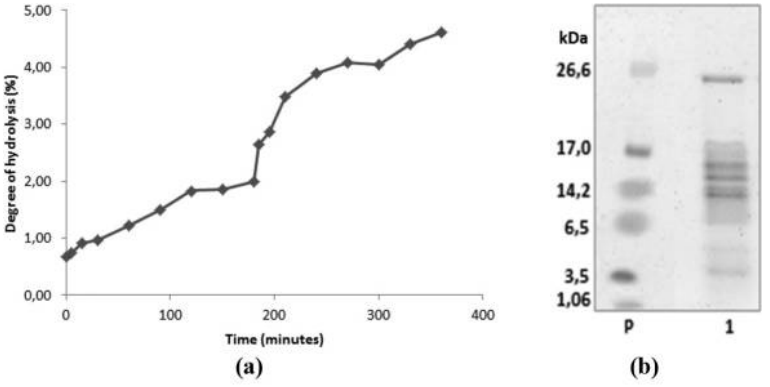
Figure 1.
(a) Chromatographic profile of the mung bean 8S globulin protein eluted on a sepharose CL 6B column. (b) SDS-PAGE of the chromatographic peak fractions of total globulin and isolated 8S globulin from sepharose CL 6B and sephadex G 200 chromatography. P. Molecular mass standard

Notes: 1. Total mung bean globulin. 2. isolated 8S globulin. 3. analysis of the 8S peak Sepharose CL 6B chromatography column. 4. analysis of the 8S peak from the Sephadex G 200 chromatography column

with pepsin and pancreatin by Tricine SDS-PAGE [Figure 2 (b)] revealed that most of the peptides had molecular weights of 6.5-18 kDa.

The total mung bean 8S hydrolysate was eluted using a Sephadex G-25 filtration column, and the fractions were assayed for the inhibition of HMG CoAr to verify the influence of mung bean vicilin on to lipid metabolism. Pravastatina, a standard drug used in the treatment of hypercholesterolemia, was used as the hypocholesterolemic control. Pravastatin resulted in a 78 per cent reduction of enzyme activity, whereas fractions 10, 12, 14, 22 and 32 of the 8S protein hydrolysate were responsible for reductions of 63.7, 64.8, 62.6, 67 and 65.5 per cent, respectively, activity of HMG CoAr enzyme (Figure 3). Although the fractions tested presented a high percentage of inhibition, the statistical analysis showed that there was no difference between the fractions tested, yet all were statistically different from the control (pravastatin) ($p < 0.001$).

Antilipidemic activity of mung bean protein has been observed previously. Yao *et al.* (2014) reported that the addition of 1 and 2 per cent of mung bean protein isolate to the feed of hypercholesterolemic hamsters led to dose-dependent reductions of serum cholesterol, triglyceride, non-HDL and hepatic cholesterol. Furthermore, there was an increase in the fecal excretion of lipids and decreased expression of HMG CoAr mRNA and CYP7A1 (cholesterol 7-hydroxylase), suggesting regulation of the HMG CoAr and CYP7A1 enzymes, to reduce cholesterol absorption and synthesis and increase the fecal



Note: 1. Total hydrolysate of mung bean 8S after 360 min

Figure 2.
(a) Degree of mung bean 8S hydrolysis after sequential digestion with pepsin and pancreatin. (b) tricine SDS-PAGE 8S after hydrolysis with pepsin and pancreatin. P. Molecular mass standard

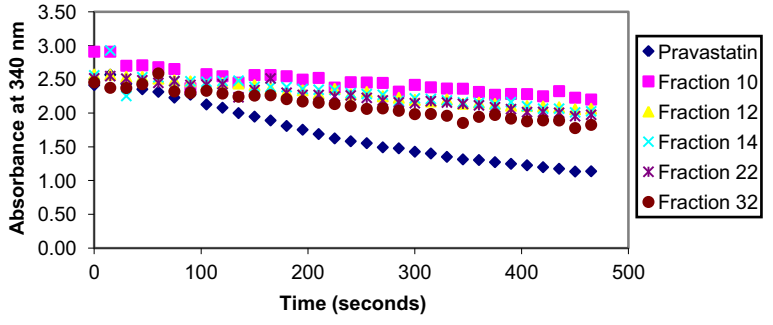


Figure 3.
Profile of HMG-CoA inhibition in the presence of mung bean 8S peptide fractions obtained by sephadex G-25 chromatography

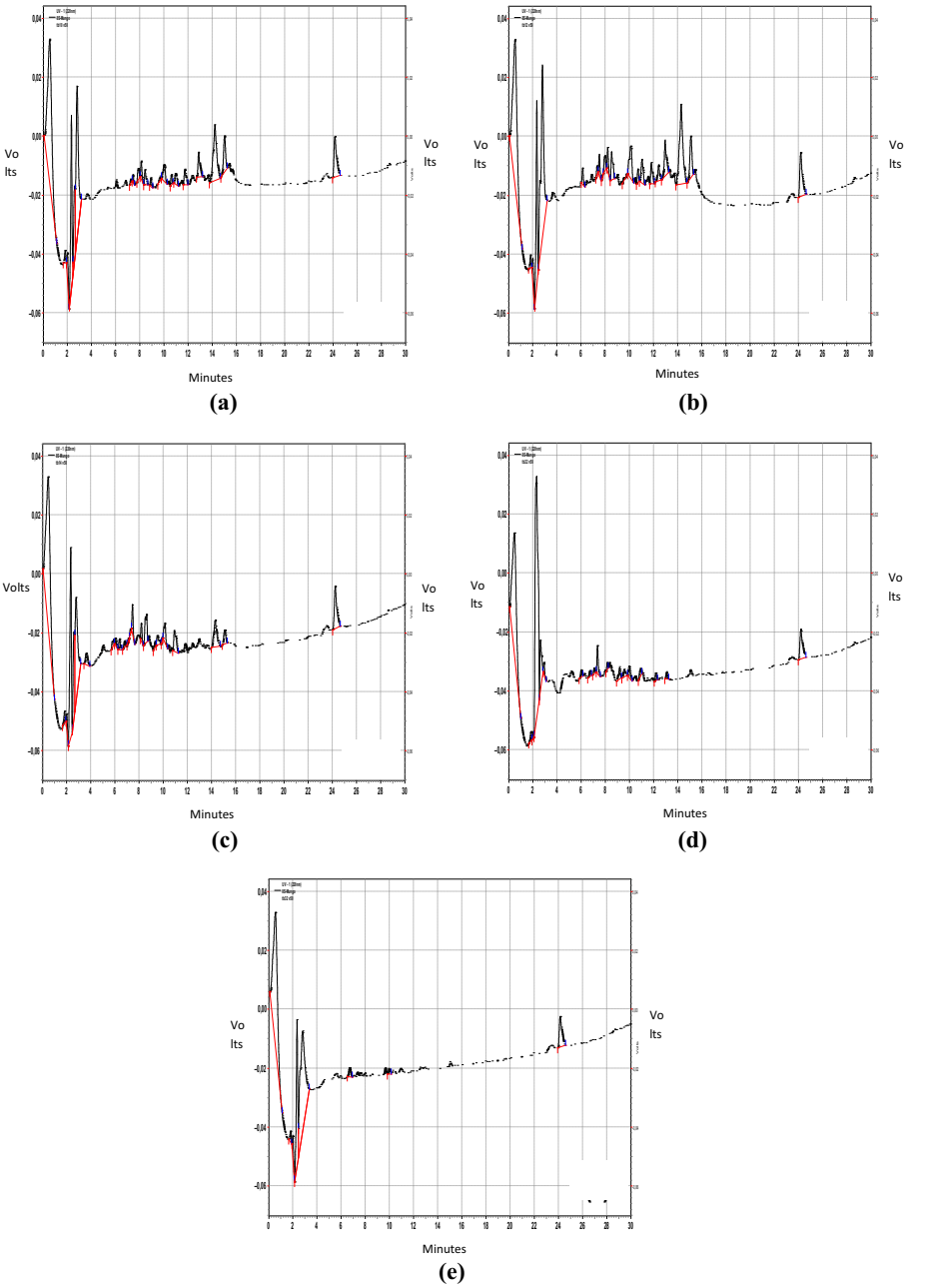


Figure 4.
HPLC Profiles of the peptide fractions with hypocholesterolemic activity

Notes: (a) Fraction 10; (b) Fraction 12; (c) Fraction 14; (d) Fraction 22; (e) Fraction 32

excretion of cholesterol. Tachibana *et al.* (2013) observed that the ingestion of a mung bean protein isolate by *Wistar* rats produced significant reductions in plasma triglycerides, cholesterol, hepatic triglycerides and insulin sensitivity, with a concomitant increase in adiponectin and reduced levels of SREBP-1 (sterol regulatory element binding factor 1), suggesting an influence of the protein isolate on the gene expression of hepatic fatty acid synthesis and stimulation of oxidation. Tachibana *et al.* attributed that the hypolipidemic effect to the amino acid composition and sequence of the protein isolate, consistent with previous studies of legumes (Moriyama *et al.*, 2004; Lovati *et al.*, 2008).

Inhibitory effects of natural (Pak *et al.*, 2005: Soares *et al.*, 2015) and synthetic (Lin *et al.*, 2015; Pak *et al.*, 2008a, 2008b, 2012) peptides on HMG-CoAr have been reported, but the underlying mechanism remained unclear. Marques *et al.* (2015) and Lammi *et al.* (2015) suggested that legume peptide inhibit HMG-CoAr via the interaction of these peptides with either the active site or NADH-binding site of the enzyme, thus preventing substrate binding to the enzyme and promoting endogenous cholesterol synthesis.

Figure 4 shows the chromatographic profiles determined by HPLC of the peptide fractions that exhibited hypocholesterolemic activity. The mean retention times, polarities and amino acids profiles of aliquots with biological activity were similar, confirming that hypocholesterolemic action is associated with the presence and chemical characteristics of certain peptides.

The 7S and 11S globulins of different species of legumes affect lipid metabolism *in vivo* and *in vitro* (Amaral *et al.*, 2014; Duranti, 2006; Ferreira *et al.*, 2015; Pak *et al.*, 2005). The amino acids sequences of the polypeptide chains and their potential structural interaction are conserved (Argos *et al*, 1985). Tang and Sun (2011) observed homology between mung bean vicilin and adzuki (*Phaseolus angularis*) vicilin, with similar secondary structure compositions. Structural immunological and sequence analysis of the N terminus of mung bean vicilin by Itoh *et al.* (2006) and Mendoza *et al.* (2001) also revealed homology between this protein, soybean β-conglycinin, phaseolin precursors and the vicilins of *Vicia faba* and pea, further supporting the hypocholesterolemic activities of mung bean vicilin observed in the present study.

4. Conclusion

The extraction and protein isolation methods were effective for obtaining mung bean vicilin. Enzymatic hydrolysis simulating the digestive process produced peptides with effects on cholesterol metabolism, including inhibition of the HMG CoAr enzyme.

Few studies have explored the biological activity of mung bean vicilin and the mechanisms underlying its observed anticholesterolemic activity are not completely understood. The peptide fractions of derived from green mung bean vicilin obtained in this work might facilitate the characterization of this protein a functional compound.

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