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In vitro and *in silico* studies of 3-hydroxy-3-methyl-glutaryl coenzyme A reductase inhibitory activity of the cowpea Gln-Asp-Phe peptide

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ABSTRACT

Previous studies have shown that cowpea protein positively interferes with cholesterol metabolism. In this study, we evaluated the ability of the fraction containing peptides of < 3 kDa, as well as that of the Gln-Asp-Phe (QDF) peptide, derived from cowpea β-vignin protein, to inhibit HMG-CoA reductase activity. We established isolation and chromatography procedures to effectively obtain the protein with a purity above 95%. *In silico* predictions were performed to identify peptide sequences capable of interacting with HMG-CoA reductase. *In vitro* experiments showed that the fraction containing peptides of < 3 kDa displayed inhibition of HMG-CoA reductase activity. The tripeptide QDF inhibits HMG-CoA reductase (IC_{50} = 12.8 μM) in a dose-dependent manner. Furthermore, *in silico* studies revealed the binding profile of the QDF peptide and hinted at the molecular interactions that are responsible for its activity. Therefore, this study shows, for the first time, a peptide from cowpea β-vignin protein that inhibits HMG-CoA reductase and the chemical modifications that should be investigated to evaluate its binding profile.

1. Introduction

Several recent studies have proposed biological effects, related to human metabolism, associated with the protein fractions of foods, especially those of plant origin (Chalamaiah, Yu, & Wu, 2018; Maestri, Marmiroli, & Marmiroli, 2016). Peptides of different sizes and physicochemical properties derived from legume seed proteins have been shown to exert various physiological activities *in vitro* and *in vivo*. For instance, effects on control of satiety (Martinez-Villaluenga, Rupasinghe, Schuler, & Mejia, 2010), on the cardiovascular system (Consonni et al., 2011; Lammi, Zanoni, & Arnoldi, 2015; Marques et al., 2015; Pak, Koo, Yun, & Kwon, 2012), on inflammatory processes (Frota, Santos, Ribeiro, & Arêas, 2015), and on cancer (Montales, Simmen, Ferreira, Neves, & Simmen, 2015) have been well established.

Clinical studies have shown changes in serum lipid profiles associated with the consumption of legume proteins. In this context, the US Food and Drug Administration (1999) recognized the action of soy protein and recommended the consumption of 25 g of protein isolate daily as a way to reduce total plasma cholesterol concentration and LDL-C, as well as reducing risk factors for cardiovascular disease.

In this context, the cowpea bean (*Vigna unguiculata* L. Walp) has been recognized for its high (180–250 g/kg) protein content (Ferreira, Amaral, Capraro, Demonte, & Zanelli, 2015; Gupta, Singh, Malhotra, Boora, & Singal, 2010), but some studies have also reported the hypocholesterolemic effect of cowpeas (Awika, & Duodu, 2016; Frota et al., 2015; Marques et al., 2015). Previous results from our research group have demonstrated a significant reduction in plasma concentrations of total cholesterol and triacylglycerides in rats fed a hypercholesterolemic diet (20% saturated fat, 1% cholesterol, and 0.5% cholic acid) when cowpea β-vignin was orally administered for 28 days (Ferreira et al., 2015).

Although many studies recognize the hypocholesterolemic activity exerted by the protein fraction of legumes (Maestri, et al., 2016), especially soybean proteins (Consonni et al., 2011; Ferreira, Silva, Demonte, & Neves, 2012, 2010; Liu, Yang, Lei, Wang, & Wang, 2017; Pak, Koo, Kasimova, & Kwon, 2005; Zhong, Liu, & Ma, 2007), the determinants and mechanisms by which these proteins exert these effects have yet to be fully elucidated. Nonetheless, some authors have

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attributed these regulatory abilities to the action of peptides, and some mechanisms of action have been proposed for their effect on cholesterol metabolism (Martinez-Villaluenga et al., 2010; Pak et al., 2012; Zhong, et al., 2007).

Notably, many studies have sought to inhibit endogenous cholesterol synthesis. Peptides consisting of 3–8 amino acid residues appear to interact within the catalytic site of 3-hydroxy-3-methyl-glutaryl coenzyme A reductase (HMG-CoA reductase), causing conformational changes that result in the loss of the activity (Pak, Koo, Kim, Yang, & Yun, 2008). A study conducted with the < 3 kDa fraction of peptides derived from the total cowpea protein isolate reported an 89% decrease in HMG-CoA reductase activity *in vitro* (Marques et al., 2015). Previous studies, carried out with soybean glycinin (11S), claim that peptides of low molecular weight generated from that protein were responsible for the HMG-CoA reductase inhibition (Pak et al., 2005; Pak, Koo, Kwon, & Kasimova, 2004). In this study, we describe, for the first time, a peptide derived from cowpea β-vignin protein with hypocholesterolemic properties. We demonstrate that this peptide is capable of modulating cholesterol synthesis by inhibiting HMG-CoA reductase *in vitro*, and also further propose how it binds within the enzyme active site, as shown via *in silico* studies.

2. Materials and methods

2.1. Botanical material and reagents

The cowpea (*Vigna unguiculata,* L. Walp) seeds were kindly provided by Dr. Rogério Faria Vieira from the Company of Agricultural Research of Minas Gerais Zona da Mata Technology Centre (Viçosa, MG, Brazil). All reagents were purchased from Sigma-Aldrich® (St. Louis, Missouri, EUA) unless otherwise specified.

2.2. Isolation and gel chromatography of β-vignin

Initially, cowpea seeds were soaked in distilled water (4 °C for 12 h), manually decorticated, air-dried (50 °C for 12 h), crushed and sieved (60 mesh), and stored at 4 °C. Furthermore, β-vignin was isolated according to methods described by Ferreira et al. (2015). Subsequently, aliquots of the isolated protein (80 mg of β-vignin) were solubilized in potassium phosphate buffer (0.05 M) at pH 7.5, NaCl (0.5 M), and sodium azide (0.01%) for chromatography on a Sepharose CL-6B gel filtration column (1.0 × 100 cm). The flow rate was 0.45 ml/min, and the protein elution was monitored by measuring the absorbance at 280 nm. The major fraction (peak tube) was dialyzed, precipitated, and lyophilized. The protein concentration was determined as described by Lowry, Rosebrough, and Farr (1951).

2.3. β-Vignin SDS-PAGE electrophoresis

Samples of total protein extract, and isolated β-vignin via chromatography were analyzed by one-dimensional electrophoresis (SDS-PAGE), as described by Laemlli (1970), using a Hoefer MiniVE electrophoresis system (Amersham Biosciences®, Hercules, CA, USA). Aliquots of 10 μg/sample were applied to the gel. Proteins with low molecular weights (between 97 and 14.4 kDa) were used as molecular markers (GE Healthcare®, Little Chalfont, United Kingdom). Gel images were analyzed by AlphaEase® software (Alpha Innotech, San Leandro, USA).

2.4. Simulation of gastrointestinal digestion and fractionation

The simulated human gastrointestinal digestion *in vitro* was performed, with minor modifications, according to procedures of sequential hydrolysis as described by Akeson and Stahmann (1964). First, the β-vignin (100 mg) was hydrolyzed by pepsin (enzyme/substrate ratio 1:66, 37 °C for 3 h, pH = 2); the pH was then neutralized, and then the hydrolyzed β-vignin was further treated with pancreatin (enzyme/substrate ratio 1:25, 37 °C for 3 h, pH = 7). The total hydrolyzed extract was ultrafiltered in a 3-kDa molecular weight cut-off membrane (MWCO), using Microcon Centrifugal Filters ultrafiltration membrane filters (Merck® Millipore, Darmstadt, Germany).

2.5. HMG-CoA reductase enzyme in vitro inhibition assay

The inhibitory activities of the fraction containing peptides of < 3 kDa, as well as the QDF peptide were probed using the HMG-CoA reductase assay kit (Sigma Aldrich, St. Louis, Missouri, USA). The experiments were carried out at 37 °C, following the manufacturer's instructions. The specific activity of the enzyme was defined as μmol of oxidized NADPH/min/mg·protein. The inhibitory properties of the peptide fraction were measured by a reduction in absorbance, which was directly proportional to enzyme activity. The results were expressed as a percentage of the control specific activity of the enzyme in the absence of pravastatin or peptide fraction.

2.6. Peptide profiling by RP-HPLC

The chromatographic profiles of the total protein hydrolysate and the fraction containing peptides of < 3 kDa were determined by high-performance liquid chromatography (HPLC), using a PerkinElmer System with a reverse phase column (C18 × 0.45 × 25 cm) and an UV/VIS detector. The gradient used was 10 min in 95% A and 50 min to achieve 25% B. The solvent system used was A (0.045% trifluoroacetic acid in ultrapure water) and B (0.036% trifluoroacetic acid in acetonitrile), with a flow rate of 1.0 ml/min, temperature of 30 °C, and readings recorded at 220 nm.

2.7. In silico screening of HMG-CoA reductase inhibitor peptides

The primary sequences of β-vignin (NCBI/GenBank Blast: AM905848 and UniProtKB: A8YQH5_VIGUN), adzuki bean 7S globulin (NCBI/GenBank Blast: AB292246.1; UniProtKB: A4PI98_PHAAN), and α-subunit of soybean β-conglycinin (NCBI/GenBank Blast: AY221105.1; UniProtKB: UniProtKB: GLCAP_SOYBN) were compared with the ClustalW server (http://embnet.vital-it.ch/software/ClustalW.html). Then, the primary sequence of β-vignin was virtually hydrolyzed by the sequential action of the enzymes pepsin (EC 3.4.23.1), trypsin (EC 3.4.21.4), and chymotrypsin (EC 3.4.21.1), as available in the BIOPEP server (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep). Afterwards, the bioactivity probability of the β-vignin-derived peptides was analyzed according to the PeptideRanker Score server (http://bioware.ucd.ie/~compass/biowareweb/Server_pages/peptide ranker.php). The hydrophobicity of peptides with a score ≥0.500 (range from 0.000 to 1.000) was calculated with the ProPAS software (version 1.1). In the sequence, they were further evaluated with the PepSite 2 server (http://pepsite2.russelllab.org) and then compared with the results from the commercial inhibitor simvastatin (PDB ID: 1HW9). The results were considered statistically significant when $p < 0.2500$ (Trabuco, Lise, Petsalaki, & Russell, 2012).

Next, peptides with a high interaction likelihood ($p \leq 0.1000$) had their interaction profile predicted by molecular docking, which was available through the AutoDock-VINA software (version 4.2.6) (Trott, & Olson, 2010). First, water molecules and ligands from the 1HW9 PDB file were discarded and hydrogen atoms were added in random orientations, as available in Sybyl 2.1-X software. Then, the PDB file was converted to a PDBQT format, using the AutoDock tools (Sanner, 1999). Subsequently, QDF and simvastatin were sketched in 2D format and then energetically minimized, using the single point optimized AM1 semi-empiric method (Keywords: 1SCF XYZ ESP NOINTER SCALE = 1.4 NSURF = 2 SCINCR = 0.4 NOMM), as implemented in the MOPAC module from SYBYL 2.1-X software. Afterwards, the search