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Introduction of the hypocholesterolemic peptide, LPYPR, to the major storage protein of mung bean [*Vigna radiata* (L.) Wilczek] through site-directed mutagenesis

[1]*Upadhyay, S. K., [1]Torio, M. A. O., [1]Lacsamana, M. S., [2]Diaz, M. G. Q., [1]Angelia, M. R. N., [1]Sucgang, A. T. and [1]Uy, L. Y. C.

[1]*Institute of Chemistry, College of Arts and Sciences, University of the Philippines Los Baños, College, Laguna 4031, Philippines*
[2]*Institute of Biological Sciences, College of Arts and Sciences, University of the Philippines Los Baños, College, Laguna 4031, Philippines*

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Abstract

The hypocholesterolemic peptide, LPYPR, was successfully introduced into the VR-1, VR-2, and VR-5 regions of the mung bean 8Sα globulin. The mutant protein (MP) has 96.69% structural homology and 97% sequence homology compared to the wild type (WT). Expression of the mutant protein in *E. coli* HMS174(DE3) was 40.66%, which was 144.42% higher than that of the WT. The WT protein and MP had MWs of about 48.4 and 48.7 kDa, respectively. These were purified using HIC and digested with trypsin. UPLC analysis of the tryptic digests of the MP revealed the successful release of the LPYPR peptide. Unlike the WT protein, cholesterol-binding capacity (mg/g sample) of the MP increased over time of tryptic digestion (average growth rate of 9.5% for crude MP and 12.5% for HIC-purified MP) for its undigested form (crude: 220.96 ± 8.65, purified: 214.71 ± 11.91), with maximum values of 380.76 ± 6.61 and 434.44 ± 10.88 were obtained for the 24-h digests of the crude and purified proteins, respectively. Similarly, the sodium taurocholate binding capacity (%) was also found to increase over time of tryptic digestion (average growth rate of 4% for crude MP and 5.67% for HIC-purified MP) for the tryptic digests of the MP. Minimum values for % bound sodium taurocholate was obtained with the undigested samples (crude: 46.71 ± 0.42, purified: 44.49 ± 0.13), while maximum values thereof were obtained with the 24-h digest samples (crude: 59.75 ± 0.30, purified 61.95 ± 0.51).

Introduction

The 8Sα globulin lacks a bioactive peptide that has hypocholesterolemic activity. However, using genetic engineering, it is possible to introduce the oligonucleotide sequence responsible for coding the hypocholesterolemic peptide, LPYPR (Takenaka *et al.,* 2000; 2001). The tool to introduce a short oligonucleotide sequence responsible for coding the bioactive peptide is called site-directed mutagenesis. This technique was previously applied to introduce methionine and cysteine residues into the 8Sα globulin of mung bean to enhance the nutritional quality of the mung bean (Torio *et al.,* 2011; 2012).

Hypocholesterolemic activity is the ability of any substance to lower serum cholesterol. Hypocholesterolemic peptides either prevent cholesterol biosynthesis by inhibiting HMG-CoA reductase (Pak *et al.,* 2007), or prevent the absorption of dietary cholesterol and reabsorption of bile salts in the gastrointestinal tract, thus leading to a decrease in serum cholesterol levels (Sugano *et al.,* 1988; Pak *et al.,* 2007; Sanossian and Ovbiagele, 2008). Hypocholesterolemic peptides can be introduced in various dietary proteins through site-directed mutagenesis, thereby enhancing nutraceutical properties.

LPYPR (MW = 644.36 Da) is one of the hypocholesterolemic peptides first derived from soybean glycinin (11S protein), and has been shown to possess a hypocholesterolemic activity that reduces the serum cholesterol in mice after oral administration (Yoshikawa *et al.,* 2000). LPYPR lowers the serum cholesterol by inhibiting the reabsorption of bile acids from the gastrointestinal tract (Yoshikawa *et al.,* 2000), as well as by inhibiting the HMG-CoA reductase (Kwon *et al.,* 2002). LPYPR interacts with bile acids not only through hydrophobic interactions but also through ionic interactions due to cationic amino acid residues such as Lys and Arg (Howard and Udenigwe, 2013).

*Corresponding author.
Email: infoshrawankupadhyay@gmail.com
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Mung bean [*Vigna radiata* (L.) Wilczek] is a leguminous crop that has about 17 - 26% globulin type storage proteins (Mendoza *et al.,* 2001; Tang and Sun, 2010). The three types of globulins in mung bean seeds are (1) the 8S vicilin type, its major storage protein; (2) the 7S basic type; and (3) the 11S legumin type; constitute about 89, 7.6, and 3.4% of the total globulins, respectively (Mendoza *et al.,* 2001; Tang and Sun, 2010). The native 8S globulin is a hetero-tetramer (MW = 200 kDa). It is composed of four different polypeptides with MWs of about 60, 48, 32, and 26 kDa as revealed by SDS-PAGE (Bernardo *et al.,* 2004).

The native 8S globulin has no disulphide linkage, and contains a carbohydrate moiety (N-linked glycoprotein) as indicated by the positive peroxidase reaction (Bernardo *et al.,* 2004). However, the carbohydrate moiety does not play any role in the assembly of the 3D structure of the protein (Bernardo *et al.,* 2004; Garcia *et al.,* 2006; Torio *et al.,* 2011). The native 8S globulin was first expressed by Bernardo *et al.* (2004) in *E. coli* HMS174(DE3), and found that the 8S globulin had three different isoforms sharing 88 to 92% homology: 8Sα, 8Sα', and 8Sβ. The cDNAs of 8Sα, 8Sα', and 8Sβ have open reading frames of 1362, 1359, or 1362, and 1559 bp, respectively; and codes for polypeptides having 454, 453, or 454, and 453 amino acid residues, respectively.

The refined native 8Sα globulin is a homotrimer of three identical subunits. Each subunit has a MW of 49 kDa (Bernardo *et al.,* 2004; Torio *et al.,* 2011), and consists of 423 amino acids. This refined native 8Sα globulin has no sulphur-containing amino acids, cysteine, and methionine. The overall structure of this 8Sα globulin closely resembles that of the soybean β-conglycinin (7S globulin) with a 68% sequence homology (Bernardo *et al.,* 2004; Itoh *et al.,* 2006). The 8Sα globulin amino acid sequence has five variable regions: (1) VR-1 near the N-terminal (1^{st} - 6^{th} amino acid), (2) VR-2 (181^{st} - 191^{st} amino acids), (3) VR-3 (214^{th} - 224^{th} amino acids), (4) VR-4 (302^{nd} - 310^{th} amino acids), and (5) VR-5 near the C-terminal (404^{th} - 423^{rd} amino acids) (Itoh *et al.,* 2006; Torio *et al.,* 2011).

Based on the study conducted by Itoh *et al.* (2006), the recombinant 8Sα globulin has a rhombohedral crystal structure with dimensions of 0.4 × 0.3 × 0.2 mm based on the diffractions obtained at resolution 2.6 Å. Studies have been conducted to enhance the nutritional quality of the mung bean by modifying the 8Sα globulin, such as by the introduction of methionine residues, sulfhydryl groups, and disulphide linkage (Torio *et al.,* 2011; 2012).

To further improve the nutraceutical value of the 8Sα globulin, the present work aimed to introduce a hypocholesterolemic LPYPR peptide and a digestion site to three different variable regions of the major storage protein of mung bean through site-directed mutagenesis, and to purify and characterise the mutant protein in terms of hypocholesterolemic activity.

Materials and methods

In silico analysis and primer design

Three of the five different variable regions of the 8Sα globulin (VR-1, VR-2, and VR-5) were chosen as the mutation sites to introduce the hypocholesterolemic peptide, LPYPR. The LPYPR peptide was introduced into three different variable regions to maximise the hypocholesterolemic activity of the resulting mutant protein (MP). These three variable regions were chosen for the introduction so that the 3D structure and function of the mutant protein will not change (Torio *et al.,* 2011; 2012). Trypsin is a pancreatic serine protease belonging to the protease of mixed nucleophile superfamily A (Haverback *et al.,* 1960). It has an optimal pH range of 7.5 to 8.5, and catalyses the cleavage of the peptide bond after arginine and lysine (Haverback *et al.,* 1960). Therefore, it was expected that trypsin will release the LPYPR peptide from the MP that had the RLPYPR sequence representing the hypocholesterolemic peptide, LPYPR, and a tryptic digestion site, R. A tryptic digestion site was added so that the hypocholesterolemic peptide (LPYPR) will be released in its intact form after treatment with trypsin. The 3D structures of the wild type (WT) and mutated 8Sα globulins were constructed using SWISS-MODEL (Biasini *et al.,* 2014). To observe the structural changes in the mutated 8Sα globulin against the WT, a 3D structural alignment was performed using the PyMOL Molecular Graphics System version 1.7.4 (DeLano, 2015) for the primer design, melting point, GC% content, and self-complementarity of the primer.

Site-directed mutagenesis

Site-directed mutagenesis of the WT 8Sα globulin was conducted followed by cloning into the pET-21d vector using the Geneart® Site-Directed Mutagenesis System (Invitrogen). The mutation was introduced in three different variable regions (VR-1, VR-2, and VR-5) of 8Sα globulin by PCR (Biometra T Optical Thermocycler®, Analytic Jena, Germany).

The mutation involving methylation followed by PCR amplification was carried out in six steps to achieve the complete introduction of the hypocholesterolemic peptide into the 8Sα globulin. In the first step, the RLPY sequence was introduced at VR-1 of the 8Sα globulin, followed by the introduction of PR

dipeptide to achieve the introduction of the complete RLPYPR sequence into VR-1. In the same way, the same RLPYPR sequence was introduced into VR-2 and VR-5. The first mutation product was used as the template DNA for the next mutation until the introduction of the entire LPYPR into all three different variable regions was achieved.

The methylation reaction was carried out for 12 min at 37°C followed by PCR. The plasmid was amplified through 16 cycles of denaturation at 94°C for 20 s, annealing at the suitable annealing temperature (Table 1) for 30 s, and elongation at 68°C for 6.5 min using the AccuPrime™ Pfx DNA polymerase (Invitrogen). The PCR products were analysed by 1% agarose gel electrophoresis (Enduro™ Electrophoresis System and EnduroTM GDS Touch, Labnet International).

The mutation was immediately followed by the recombination reaction to cleave the methylated original plasmid template. A mutated plasmid carrying RLPYPR at three different variable regions was then transformed into the competent *E. coli* HMS174(DE3). Following transformation, the WT plasmid carrying the mutated 8S globulin was isolated, and confirmed by DNA sequencing (AIT Biotech, Singapore).

Protein expression of WT and mutated 8Sα globulin

Fifty millilitres of the overnight-grown culture of the *E. coli* HMS174(DE3) transformants carrying the WT and the mutated 8Sα globulin cloned into the pET-21d vector were inoculated in 200 mL of sterilised LB broth containing 0.1 mg/mL ampicillin, and was incubated for 4 h at 37°C with constant shaking. After 4 h of incubation, 50 mL of 0.2 µm filter-sterilised 10 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture. After that, the culture was incubated for 4 d at 25°C with constant shaking.

After incubation, cells were harvested by centrifugation (Hermle Z326K) at 6,000 rpm for 15 min at 4°C. The cell pellets were suspended in 6 mL of buffer-A (Mendoza *et al.,* 2001). Buffer-A was made up of 35 mM phosphate buffer of pH 7.6 that contained 1 mM EDTA, 0.4 M NaCl, 0.1 M p-amidinophenyl-methylsulfonyl-fluoride (APMSF), 1 µg/mL leupeptin, and 1 µg/mL pepstatin-A. The cell suspension was ground for 5 min in liquid nitrogen using a mortar and pestle over ice to break the cell wall. The ground suspension was centrifuged (Hermle Z326K) at 12,000 rpm for 15 min at 4°C. Unless otherwise mentioned, all of the steps were performed at 4°C.

Purification of the WT and mutated 8Sα globulins

Before performing the hydrophobic interaction chromatography, hydrophobicity of the WT and mutated 8Sα globulins was determined using an online tool, Peptide 2.0 Inc. Four millilitres of each crude extract (the WT and the mutated 8Sα globulins) was saturated with ammonium sulphate (35% final concentration) followed by centrifugation (Hermle Z326K) at 10,000 rpm for 10 min at 4°C. The respective supernatants of the WT and the mutated 8Sα globulins were collected and subjected to hydrophobic interaction chromatography using Macro-Prep® t-Butyl HIC Resin (Bio-Rad). The column was washed with three column volumes of buffer-A, followed by equilibration with three column volumes of buffer-A containing 35% ammonium sulphate. After

Table 1. A list of primers containing mutated oligonucleotide sequences for the introduction of LPYPR in different variable regions of the 8Sα globulin through site-directed mutagenesis. The light grey shaded area represents the tryptic digestion site, while the dark grey shaded area represents the site of hypocholesterolemic peptide, LPYPR.

Sites	Substituted AA	Oligonucleotide sequence	Melting Temperature (°C)	Annealing temperature (°C)
VR-1	RLPY	5'-TACAATACTATGGTGCGTCTGCCGTATCCACTGCTGCTGTTG-3'	69	62.6
		3'-ATGTTATGATACCACGCAGACGGCATAGGTGACGACGACAAC-5'	69	62.6
	RLPYPR	5'-GTGCGTCTGCCGTATCCGCGTCTGCTGTTGCTGGGA-3'	72	67.2
		3'-CACGCAGACGGCATAGGCGCAGACGACAACGACCCT-5'	72	67.2
VR-2	RLPY	5'-ACAGAAGCACAACAACGTCTGCCGTATGGATTCAGCAAGAAT-3'	67	62.1
		3'-TGTCTTCGTGTTGTTGCAGACGGCATACCTAAGTCGTTCTTA-5'	67	62.1
	RLPYPR	5'-CAACGTCTGCCGTATCCGCGTAGCAAGAATATTCTA-3'	66	59.3
		3'-GTTGCAGACGGCATAGGCGCATCGTTCTTATAAGAT-5'	66	59.3
VR-5	RLPY	5'-GAGATACCTACAGAGCGTCTGCCGTATACGTTCCCTGCGTCT-3'	71	63.5
		3'-CTCTATGGATGTCTCGCAGACGGCATATGCAAGGGACGCAGA-5'	71	63.5
	RLPYPR	5'-GAGCGTCTGCCGTATCCGCGTCCTGCGTCTGGTGAG-3'	74	66.8
		3'-CTCGCAGACGGCATAGGCGCAGGACGCAGACCACTC-5'	74	66.8

equilibration, the protein sample was applied to the column. The protein of interest was eluted with 55 mL of a linear gradient of decreasing ammonium sulphate (35 - 0%) in buffer-A.

One millilitre per fraction was collected, and the absorbance of each fraction was determined using UV/Visible Spectrophotometer (Thermo Scientific™ Multiskan™ GO) at 280 nm. Fractions under each peak were pooled and dialysed against buffer-A containing 0.1 M NaCl. The dialysed samples were concentrated using polyethylene glycol 3350 (PEG).

Protein quantification of the WT and mutated 8Sα globulins

The protein contents of the crude protein, HIC-purified WT protein, and MP were determined using the method of Bradford (1976) in which bovine serum albumin was used as the standard.

SDS-PAGE

Crude extracts, as well as the HIC-purified WT protein and MP samples, were analysed by SDS-PAGE (Laemmli, 1970) using 11% resolving gel. Ten micrograms of each crude sample and 5 µg of each HIC-purified sample of WT protein and MP were mixed with the SDS sample buffer containing β-mercaptoethanol, and were loaded into the SDS-PAGE. The protein markers (MW = 10 - 250 kDa) used were: (1) Precision Plus Protein™ Unstained Standards, and (2) Pre-stained Precision Plus Protein™ All Blue Standards. The electrophoresis was carried out at 110 V. After electrophoresis, gels were stained with a staining solution containing 0.05% (w/v) CBB R-250 in 50% (v/v) methanol, 40% (v/v) distilled water, and 10% (v/v) acetic acid. After 2 to 3 h of staining, the gels were destained with a solution containing 50% (v/v) methanol, 40% (v/v) distilled water, and 10% (v/v) acetic acid.

The relative MWs of the WT protein and the mutated 8Sα globulin were estimated by scanning the SDS-PAGE gels using the gel analysis software Image Lab™ v6.0.0 built 25 (Bio-Rad).

Enzymatic digestion of WT and mutated 8Sα globulins

The crude extracts, as well as the HIC-purified WT and the mutated 8Sα globulins were subjected to tryptic digestion. The protein sample (final concentration of 0.3 mg/mL) and trypsin (final concentration of 25 µg/mL) were mixed at a proportion of 20:1 (w/w). The digestion was carried out at 37°C for 24 h. An aliquot of 200 µL was collected immediately after adding trypsin. Subsequent aliquots were collected successively at 2, 6, 12, and 24 h after adding trypsin. Each aliquot was boiled for 5 min in the boiling water bath to deactivate the trypsin. Each digest was analysed by SDS-PAGE using 11% resolving gel, and hypocholesterolemic activity for each was determined according to the method of Bangoura *et al.* (2009) and Kongo-Dia-Moukala *et al.* (2011).

UPLC-based LPYPR mapping of tryptic digests of the MP

The 24-h tryptic digests of the HIC-purified mutated 8Sα globulin were subjected to UPLC analysis to map the peptide fragments and LPYPR released during digestion. The UPLC-based peptide mapping was carried out just for the qualitative analysis primarily to detect the presence of the LPYPR peptide in the tryptic-digest of the mutated 8Sα globulin. The chemically synthesised LPYPR dissolved in distilled water (5 mg/mL) was used as the standard to determine the retention time of the peptide. The samples and the LPYPR standard were eluted at a flow rate of 0.2 mL/min using the RP C_{18} column with dimension of 2.1 × 50 mm and particle size of 1.7 µm (Acquity Arc System, Waters). The elution solvents used were the A-solvent (0.1% TFA in ultra-high-quality water) and B-solvent (80% acetonitrile in ultra-high-quality water, 0.1% TFA). The UPLC program was set to 2% B-solvent for initial conditioning, followed by a linear gradient of 2 to 60% B-solvent for 3.4 min, and then 60 to 0% B-solvent for 3.4 to 3.63 min, followed by 0% B-solvent until the end of the run. The elution was monitored at 214 nm to map the peptide fragments and the LPYPR peptide.

In vitro cholesterol micellar solubility inhibition

The *in vitro* cholesterol micellar solubility inhibition of the undigested and digested samples of the crude protein, as well as the HIC-purified WT and mutated 8Sα globulins were performed according to the method of Bangoura *et al.* (2009). The chemically synthesised LPYPR and the bile acid sequestrant, cholestyramine (final concentration of 1 mg/mL), were used as positive controls, and cholesterol micellar solution without a sample was used as negative control to calculate the recovery. Twenty-five millilitres of the cholesterol micellar solution were prepared to contain 10.0 mM sodium taurocholate, 0.4 mM cholesterol, 1.0 mM oleic acid, and 132 mM NaCl in 15 mM sodium phosphate (pH 7.4). The micellar solution was prepared by sonication for 5 min at an output frequency of 20 kHz and temperature of 37°C (Omni Sonic Ruptor 400).

To each 400 µL of the cholesterol micellar solution, 50 µL of buffer (15 mM sodium phosphate

buffer of pH 7.4) and 50 µL of the tryptic digest were added. The mixtures were incubated for 24 h at 37°C. Then, the samples were centrifuged at 14,000 rpm for 30 min at 37°C (Hermle Z326K). The supernatant was collected from each sample and the cholesterol content was measured according to the method of Brown *et al.* (1954).

Eighty microliters of the supernatant from each sample was loaded into a 96-well microplate in triplicates. Glacial acetic acid (100 µL) was added to each sample to obtain a final volume of 180 µL. To each of the samples, 120 µL of the colour reagent consisting of 0.1% (w/v) $FeCl_3.6H_2O$ and 1% (v/v) glacial acetic acid in concentrated sulfuric acid (Brown *et al.,* 1954) was added slowly and mixed into the solution thoroughly. For the negative (without sample) control, 80 µL of cholesterol micellar solution was added. After 15 min of incubation, the absorbance reading was measured at 560 nm, and the cholesterol content of each sample was determined using a standard calibration curve generated from the 0 to 48 µg/mL cholesterol standard solutions prepared in glacial acetic acid. The cholesterol-binding capacity of the sample was expressed as milligrams of bound cholesterol per gram of sample.

In vitro bile acid-binding capacity

The *in vitro* bile acid-binding capacities of the undigested and digested samples of the crude as well as of HIC-purified WT and mutated 8Sα globulins were determined according to the method of Hu *et al.* (2008) with slight modification (Kongo-Dia-Moukala *et al.,* 2011). A 2.0 mM sodium taurocholate solution was prepared in 50 mM phosphate buffer (pH 6.5). The chemically synthesised LPYPR and cholestyramine with the final concentration of 1.0 mg/mL were used as positive controls, and 2.0 mM sodium taurocholate solution without a sample was used as the negative control to calculate the recovery.

To each sample, 400 µL of 2 mM sodium taurocholate solution, 50 µL of buffer (50 mM phosphate buffer of pH 6.5), and 50 µL of the sample were added. The mixture was incubated for 1 h at 37°C in a shaking water bath. After incubation, the sample was centrifuged at 12,000 rpm for 20 min at 10°C (Hermle Z326K). The supernatant was collected in a new set of microcentrifuge tubes. The sodium taurocholate concentration in each sample was determined by UPLC using an RP C_{18} column (2.1 × 50 mm with the particle size of 1.7 µm) (Acquity Arc System, Waters).

Three injections of each sample at 10 µL per injection were done. Samples were eluted with 80:20 methanol solution (0.04% KH_2PO_4,) at a flow rate of 0.2 mL/min for 3 min. The absorbance was monitored at 210 nm, and the peak areas were used for the calculation of the concentration of unbound bile acids. The sodium taurocholate concentration in each sample was calculated using the standard calibration curve generated from 0, 400, 800, 1200, 1600, and 2000 µM of sodium taurocholate solutions. The % bound sodium taurocholate was determined using the formula: % bound bile acids = $[(C_c-C_s)/C_c] \times 100$, where C_c and C_s were the bile acid concentrations in the control and the sample, respectively.

Statistical analysis

The significance of differences among the groups (WT protein vs. MP) and within the groups (digestion time) was determined by Tukey's HSD test after one-way ANOVA using the Statistical Tool for Agricultural Research (STAR) package (International Rice Research Institute, Los Baños, Laguna, Philippines). The significance was defined at a confidence interval of 95%, and the data were expressed as mean ± SEM.

Results and discussions

In silico analysis and primer design

The mutation sites were chosen so that the 3D structure of the protein would not be affected. A comparison of the 3D structure of the WT and mutated 8Sα globulins was carried out using the PyMOL Molecular Graphics System version 1.7.4 (DeLano, 2015). These two proteins shared 96.69% structural homology.

The DNA sequence alignment of the WT and mutated gene with the complete introduction of the RLPYPR sequence at three different variable regions of VR-1, VR-2, and VR-5 was carried out using the Jalview version 2 program (Waterhouse *et al.,* 2009). The WT and mutated 8Sα globulins shared 97% sequence homology.

Site-directed mutagenesis

The purity and concentrations of the isolated plasmids containing the WT and the mutated 8Sα globulins were calculated using the UV/Visible Spectrophotometer (Thermo Scientific™ Multiskan ™ GO). Purities of the plasmids containing the WT and mutated 8Sα globulins were 1.88 and 1.86, respectively, while their concentrations were 0.2 and 0.21 mg/mL, respectively.

Expression of the WT and mutated 8Sα globulins

The extraction buffer contained various types of protease inhibitors such as (1) EDTA for metalloprotease inactivation, (2) *p*-amidinophenyl-methylsulfonyl-fluoride for serine protease inactivation, (3) leupeptin for cysteine, serine, and threonine peptidases inactivation, and (4) pepstatin-A for aspartyl protease inactivation. Proteases are present in various membrane vesicles inside the cell, and when cells are disrupted, they come out into the crude extracts together with all of the other contents and could hydrolyse the protein of interest. Therefore, protease inhibitors were necessary for the extraction buffer to prevent the degradation of the protein of interest during the extraction process.

Using the gel analysis software Image Lab™ v6.0.0 built 25 (Bio-Rad), the expression of the mutated 8Sα globulin was calculated to be 40.66%, which was about 144.42% higher than that of the WT 8Sα globulin (28.15%). However, expression of the WT and the mutated 8Sα globulins normalised against the total proteins were both 0.3 mg/mL. The higher expression (40.66%) of the mutated 8Sα globulin might be due to the lower expression of the contamination proteins. In contrast, the WT 8Sα globulin expression was low (28.15%), and this could be due to the expression of other contaminating proteins. These data were calculated using the results of the densitometric analysis of the SDS-PAGE for the crude samples of WT and mutated 8Sα globulins.

Purification of the WT and mutated 8Sα globulins

HIC separates different protein samples based on their hydrophobicity, a self-association character of a protein in an aqueous solution (McCue, 2009). The hydrophobicity of the WT and the mutated 8Sα globulin was enhanced by adding ammonium sulphate (35% final concentration), thus allowing protein purification possible by HIC. The HIC purification profile of the WT and the mutated 8S globulins are shown in Figure 1. The second peak corresponds to the 8Sα globulin. The WT and the mutated 8S globulins were successfully purified and found to have the same retention volumes (35 mL). This may be due to the similarity of their hydrophobicity values of 40.53 and 40.31%, respectively, as calculated using an online tool, Peptide 2.0 Inc.

Protein quantification of the WT and mutated 8Sα globulins

The total protein concentrations of the crude and HIC-purified WT and mutated 8Sα globulins were calculated using the Bradford assay. The protein concentrations of the crude WT, crude MP, HIC-purified WT, and HIC-purified MP were 1.06 ± 0.01, 0.79 ± 0.01, 0.26 ± 0.01, and 0.27 ± 0.01 mg/mL, respectively. The decreasing trend in total protein concentrations after purification indicated the successful purification of the 8Sα globulin in both the WT and mutated samples.

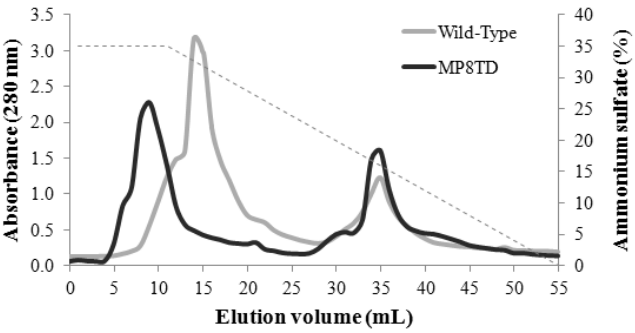

Figure 1. A chromatogram showing HIC purification of the WT and the mutated 8Sα globulins. Proteins were eluted at the same retention volume (35 mL) using the decreasing gradient of 35 - 0% ammonium sulphate in 35 mM phosphate buffer 0.4 M NaCl and 1 mM EDTA (pH 7.6).

SDS-PAGE analysis

With the help of densitometric analysis of the SDS-PAGE gel carried out using the gel analysis software Image Lab™ v6.0.0 built 25 (Bio-Rad), the relative MWs of the WT and mutated 8Sα globulins were calculated to be 48.4 and 48.7 kDa, respectively. The observed MWs for the WT and mutated 8Sα globulins are in agreement with the previously reported MW of 49 kDa for the native 8Sα globulin (Bernardo *et al.,* 2004; Torio *et al.,* 2011).

SDS-PAGE analysis of the tryptic digest of the HIC-purified WT and mutated 8Sα globulins revealed complete digestion of the protein as indicated by the absence of bands after the addition of the trypsin.

UPLC-based LPYPR mapping of the tryptic digests of the mutated 8Sα globulin

UPLC-based LPYPR peptide mapping of 24-h digests of the HIC-purified MP gave five peaks at 214 nm, one of which corresponded to the LPYPR. The retention time of the chemically synthesised LPYPR was determined to be 1.65 min and was used to correlate the possible peak for the LPYPR in the tryptic digest of the mutated protein. As shown in Figure 2, the peak at 1.73 min of retention time (which is close to 1.65 min) in the 24-h tryptic digest of the MP confirms that the LPYPR has been

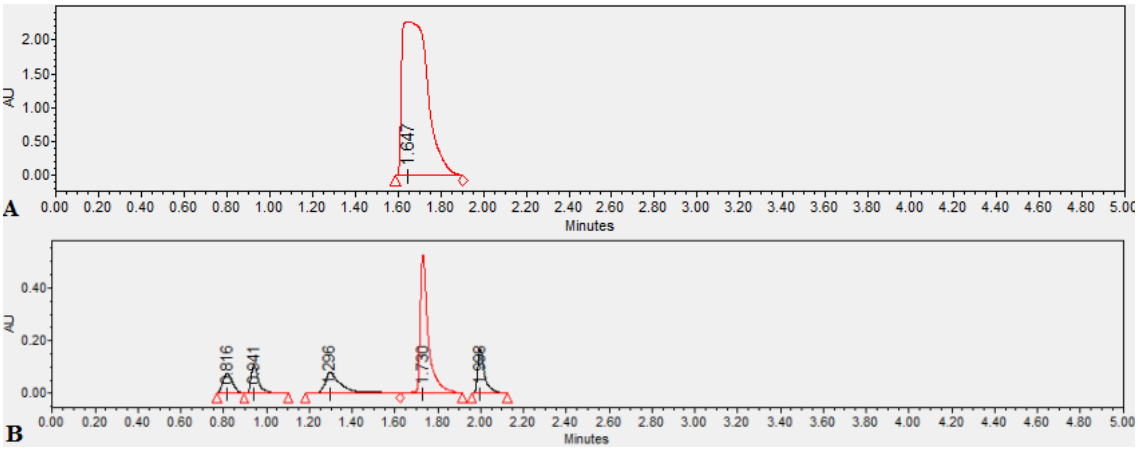

Figure 2. UPLC-based peptide mapping of (A) chemically synthesised LPYPR, and (B) pooled sample of 12- and 24-h tryptic digests of the MP showing the release of LPYPR during the tryptic digestion.

successfully released from the MP after trypsin treatment.

In vitro cholesterol micellar solubility inhibition

The cholesterol-binding capacities of the undigested and digested crude samples, as well as the HIC-purified samples of the WT protein and MP are presented in Figure 3. As shown in the Figure 3, the cholesterol-binding capacity of the crude WT protein was found to be maximum (269.64 ± 10.81 mg/g) for the 24-h tryptic digest which was significantly different from the cholesterol-binding capacities of the undigested sample of the crude WT protein as well as of the positive controls, LPYPR, and

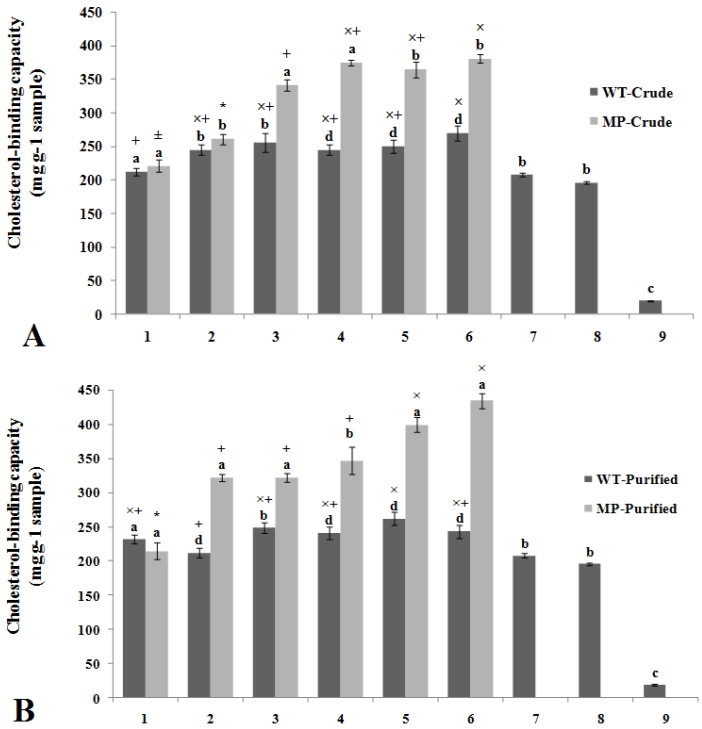

Figure 3. Cholesterol-binding capacities of the (A) crude samples, and (B) HIC-purified samples, their tryptic digests, and positive controls. Legend description for x-axis: (1) undigested samples; (2) an aliquot of samples taken immediately after the addition of trypsin; (3), (4), (5), and (6) aliquots of the sample taken at 2, 6, 12, and 24 h of trypsin treatment, respectively; (7) LPYPR as positive control 1; (8) cholestyramine as positive control 2; (9) negative control. The significance of differences was analysed by Tukey's HSD test after one-way ANOVA at 95% confidence interval. Values are mean with bars indicating SEM. Means with similar letter are not significantly different in group-wise comparison. Means with similar symbols are not significantly different in digestion time-wise comparison.

cholestyramine. However, in the case of HIC-purified WT protein, no significant differences were observed among the cholesterol-binding capacities of the undigested sample and the tryptic digests.

The cholesterol-binding capacities of the crude MP and the HIC-purified MP have linearly increasing trends over time of tryptic digestion with minimum values of 220.96 ± 8.65 and 214.71 ± 11.9 mg/g for the undigested crude MP and HIC-purified MP, respectively, and maximum values of 380.76 ± 6.61 and 434.44 ± 10.88 mg/g for the 24-h tryptic digests of the crude MP and HIC-purified MP, respectively.

The linearly increasing cholesterol-binding capacities of the crude MP and HIC-purified MP over time of digestion indicated that the LPYPR peptide has been released from the protein during digestion, as shown by UPLC-based LPYPR mapping of the 24-h tryptic digest of the MP (Figure 2).

Finally, the cholesterol-binding capacity of the (1) undigested MP and (2) undigested and trypsin-digested WT protein of crude and HIC-purified samples might be due to hydrophobic interactions of yet unknown mechanisms (Amaral *et al.,* 2017).

The LPYPR released during tryptic digestion competes with the cholesterol for the formation of cholesterol micellar solution, and also interacts with the bile acids through hydrophobic and ionic interactions (Yust *et al.,* 2012; Zhang *et al.,* 2012; Howard and Udenigwe, 2013), thereby disrupting the cholesterol micelle. This disruption or prevention of cholesterol micellar formation leads to the aggregation of cholesterol that eventually gets removed from the gastrointestinal tract through defecation.

In vitro bile acid-binding capacity

The percentage of sodium taurocholate binding capacities of the crude and HIC-purified WT protein and MP are presented in Figure 4. As shown in Figure 4, sodium taurocholate binding capacity of the crude WT protein was found to be maximum (45.14 ± 0.38%) for the 24-h post-digestion which was significantly different from that of the undigested form of crude WT protein. In the same way, sodium taurocholate binding capacity of the

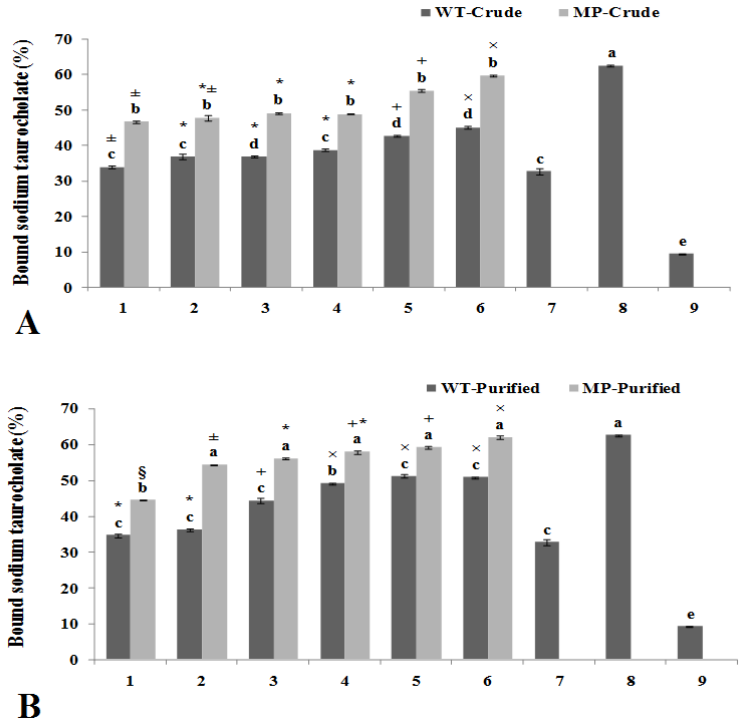

Figure 4. Sodium taurocholate binding capacities of the (A) crude samples, and (B) HIC-purified samples of mung bean 8Sα globulin, their tryptic digests, and positive controls. Legend description for x-axis: (1) undigested samples; (2) an aliquot of samples taken immediately after the addition of trypsin; (3), (4), (5), and (6) aliquots of the sample taken at 2, 6, 12, and 24 h of trypsin treatment, respectively; (7) LPYPR as positive control 1; (8) cholestyramine as positive control 2; (9) negative control. The significance of differences was analysed by Tukey's HSD test after one-way ANOVA at 95% confidence interval. Values are mean with bars indicating SEM. Means with similar letter are not significantly different in group-wise comparison. Means with similar symbols are not significantly different in digestion time-wise comparison.

HIC-purified WT protein was maximum (51.16 ± 0.49%) for the 12-h post-digestion which was significantly different from that of the undigested form of the HIC-purified WT protein.

As in the case of cholesterol-binding capacities, the *in vitro* % sodium taurocholate binding capacities of the crude and HIC-purified MP were found to have linearly increasing trends over time of tryptic digestion. For crude MP, the maximum % sodium taurocholate binding capacity was observed at the 24-h post-digestion (59.75 ± 0.30%). In the same way, the HIC-purified MP had the maximum % sodium taurocholate binding capacity at 24-h post-digestion (61.95 ± 0.51%). The % bound sodium taurocholate at 24-h post-digestion of the crude and HIC-purified MP were comparable with one of the positive controls, cholestyramine, and significantly higher than the other positive control, the chemically synthesised LPYPR peptide.

The higher % bound sodium taurocholate capacities of the tryptic digests of the crude and HIC-purified MP is related to the release of LPYPR during digestion. The released LPYPR interacts with the sodium taurocholate via ionic and hydrophobic interactions (Yust *et al.,* 2012; Zhang *et al.,* 2012; Howard and Udenigwe, 2013) that eventually gets removed from the gastrointestinal tract through defecation, thereby, lowering the reabsorption of the bile acids from the gastrointestinal tract. Though both the WT protein and the MP showed bile acid-binding capacities, MP had higher bile acid-binding activity, thus indicating the successful release of the LPYPR peptide during tryptic digestion.

Conclusion

The hypocholesterolemic peptide, LPYPR, was successfully introduced into three different variable regions, VR-1, VR-2, and VR-5 of the WT 8Sα globulin of mung bean. The WT protein and MP were successfully expressed in *E. coli* HMS174(DE3) with 28.15 and 40.66% expression, respectively. The two expressed proteins were successfully purified through HIC, and were trypsin-digested. LPYPR was successfully released from the MP, and was responsible for the higher hypocholesterolemic activity of its tryptic digest. The cholesterol-binding and % sodium taurocholate-binding capacities of the MP were significantly improved over those obtained for the WT protein (confidence interval of 95%). It is possible to explore the potential use of the MP as a dietary supplement that can bind cholesterol, thereby, lowering serum cholesterol level, and bind bile acids to prevent their reabsorption from the gastrointestinal tract.

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