



Effects of engineered methionine in the 8S α globulin of mungbean on its physicochemical and functional properties and potential nutritional quality

Mary Ann O. Torio^{a,b}, Motoyasu Adachi^{c,1}, Roberta N. Garcia^a, Krisna Prak^c, Nobuyuki Maruyama^c, Shigeru Utsumi^{c,2}, Evelyn Mae Tecson-Mendoza^{a,*}

^a Institute of Plant Breeding, Crop Science Cluster, College of Agriculture, University of the Philippines Los Baños, College, Laguna, 4031 Philippines

^b Institute of Chemistry, College of Arts and Sciences, University of the Philippines Los Baños, College, Laguna, 4031 Philippines

^c Laboratory of Food Quality Design and Development, Graduate School of Agriculture, Kyoto University, Uji, Kyoto 611-0011, Japan

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ABSTRACT

The major storage protein of mungbean, 8S α globulin or vicilin, was engineered using site-directed mutagenesis to increase the number of methionine (Met) residues in the molecule for improvement of functional and nutritional qualities. Eight Met-rich proteins were designed and prepared to have 2 to 10 Met residues introduced in disordered regions II and IV. The designed proteins were highly expressed as soluble form in *Escherichia coli*. Their production level of the modified proteins was estimated to be about 30%, and was almost the same as that of 8S α globulin wild type (WT). The modified proteins formed stable native conformation similar to WT as shown by gel filtration chromatography. They demonstrated greater stability in terms of thermal denaturation temperature and greater emulsifying ability and emulsion stability, especially the 10-Met protein, compared to the wild type. Met-rich proteins with 3, 5, and 10 Met residues had 74, 96, and 145% of nutritional requirement for Met compared with 41% for WT. Based on allergenicity prediction programs, WT and all the modified proteins had no allergenic potential.

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1. Introduction

Storage proteins of legumes, in general, lack the sulfur-containing amino acids methionine (Met) and cysteine (Cys). Protein engineering has been considered as a potentially important tool in the improvement of proteins of important food crops such as soybean and mungbean in terms of nutritional and functional qualities (Altenbach, Pearson, Meeke, Staraci, & Sun, 1989; Hoffman, Donaldson, & Herma, 1988; Kim, Kamiya, Kanamori, Utsumi, & Kito, 1990; Utsumi, 1992). Soybean proteins consist primarily of glycinin, the 11S globulin, (40%) and β -conglycinin, the 7S globulin or vicilin (30%). In the absence of data on the three-dimensional structures of soybean glycinin, Kim et al. (1990) introduced five contiguous Met residues in variable regions identified based on the alignment of amino acids with those of known glycinin-type globulins from legumes and other crops. The resulting modified proteins were successfully expressed in *E. coli* in high amounts and in soluble form and were also crystallized. The modified

glycinins containing IV + 4 Met and V + 4 Met were introduced and found to accumulate in tobacco (Takaiwa et al., 1995; Utsumi et al., 1993), rice grains (Katsube et al., 1999) and potato tubers (Utsumi et al., 1994) similar to the wild type glycinin as reviewed by Utsumi, Katsube, Ishige, and Takaiwa (1997). Soybean glycinin (11S) has also been successfully engineered to introduce sulfhydryl groups and disulfide linkages, and their effects on the structural stability and heat-induced gelation had been reported (Adachi, Chunying, & Utsumi, 2004).

Mungbean (*Vigna radiata* (L.) Wilczek L.) is a popular grain legume in Asia. Its major storage protein is the vicilin or 8S globulin at 89%, while the 7S basic globulin and 11S globulin comprise the remaining 11% (Tecson-Mendoza, Adachi, Bernardo, & Utsumi, 2001) in contrast to the storage protein profile of soybean. The major storage protein, the vicilin type (8S), has a native molecular weight of 200,000, no disulfide linkage, possesses carbohydrate moiety and cross-reacts with soybean anti- β -conglycinin. Further study revealed that mungbean vicilin has three isoforms, 8S α , 8S α' and 8S β (Bernardo et al., 2004). The 8S α cDNA was cloned and its protein crystallized forming a rhombohedral structure (Bernardo et al., 2004). Garcia, Adachi, Tecson-Mendoza, Bernardo, and Utsumi (2006) showed that the presence of N-linked glycans was not essential in the assembly, stable conformation and emulsifying ability of the mungbean vicilin. The three dimensional structure of the 8S α globulin of mungbean was established for the first time by X-ray crystallography (Itoh et al.,

* Corresponding author at: Tel.: +63 49576 0025; fax: +63 49536 5287.

E-mail addresses: emtmdoza@nast.ph, emtphil@yahoo.com

(E.M. Tecson-Mendoza).

¹ Present address: Quantum Beam Science Directorate, Japan Atomic Energy Agency, Tokaimura, Ibaraki 319-1195 Japan.

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2006). The refined 8S α globulin is a trimer of three identical subunits with a molecular mass of about 49 kDa. It has five variable regions (disordered regions): the N-terminal (amino acids 1–6), internal I (amino acids 181–191), internal II (amino acids 214–224), internal III (amino acids 302–310) and C-terminal (amino acids 404–423).

Based on the derived amino acid sequence of the mungbean 8S α globulin (Bernardo et al., 2004), this protein has 4 Met residues per subunit which corresponds to about 40% amino acid score. Reported mungbean amino acid scores range from 32% (Tsou, Hsu, Tan, & Park, 1979) to 46% (Khalil, 2006) with the sulfur amino acids cysteine and methionine as limiting amino acids. Improving the nutritional quality of mungbean by increasing its Met content will greatly contribute to improving the diet of many people in developing countries where mungbean serves as an important source of dietary protein. In addition, enhancing functional properties such as emulsification, foaming and others, is very important in increasing the application of mungbean proteins in processed food.

In line with the efforts to improve the nutritional value and functional properties of mungbean, this study aimed to engineer additional Met residues in the 8S α globulin through site-directed mutagenesis and to purify and characterize the Met-rich modified proteins. It is important that the resulting Met-rich proteins form the correct and stable conformation and be expressed as soluble protein at a high level. Further, we determined some physicochemical and functional properties and since the resulting Met-rich proteins are novel proteins, we also studied their potential nutritional quality in terms of chemical score and allergenicity.

2. Materials and methods

2.1. Modeling of disordered regions

The suitable sites for engineering methionine specifically within the disordered regions (Fig. 1) were selected based on the three dimensional structure of 8S α globulin (Itoh et al., 2006). The models for wild type (WT, the unmodified recombinant protein) and modified proteins were built by the program Loopy (Xiang, Soto, & Honig, 2002) to predict the structure in the disordered loop regions. After generation of hydrogen atoms by the program CNS (Brunger et al., 1998), the electrostatic energy of the modeled structures was calculated by the program Delphi (Rocchia et al., 2002). At first, a disordered region of a protomer in the WT trimer molecule of the biologically active form was modeled by Loopy, and then, its modeled protomer was used to generate the trimer molecule. As a result, all three protomers included the modeled disordered region and the generated trimer was subjected to electrostatic calculation.

2.2. Site-directed mutagenesis for addition and substitution of methionine residues

Site-directed mutagenesis of the cloned 8S α globulin in pET-21d vector was done by PCR (Mini MJ Research and MyCycler, BioRad, U.S.A.) using the Gene-Tailor site-directed mutagenesis kit by Invitrogen (USA). Mutation was done at sites 2 and 4 disordered regions of the 8S α

globulin in mungbean (Fig. 1). Primers as listed in Table 1 were designed, synthesized and used. Plasmid template was amplified by 30 cycles of denaturation at 94 °C for 30 s, annealing at T_m of the primer for 30 s and elongation at 68 °C for 10 min using High Fidelity DNA Taq Polymerase (Invitrogen). The resulting DNA fragment of 6500 bp which corresponds to the length of the cDNA of mungbean vicilin 8S α subunit and pET-21d vector was used for transfection into *E. coli* DH5 α strain. Plasmids of the mutants were isolated and submitted to Macrogen (Seoul, Korea) for sequencing.

2.3. Protein expression

The expression plasmids were transfected into *E. coli* HMS174 (DE3). One milliliter of an overnight culture was inoculated in 500 mL of LB medium containing 50 μ g/mL carbenicillin and incubated at 37 °C until OD₆₀₀ of about 0.4 to 0.8. At this point, isopropyl- β -D-thiogalactopyranoside (IPTG) was added to the culture at a final concentration of 1 mM. The cells were grown further for 48 h at 20 °C and harvested by centrifugation at 6700 g for 30 min at 4 °C. The cells were resuspended in buffer A (2.0 g cells/100 mL buffer) and sonicated. Buffer A used in the extraction and purification experiments consisted of 35 mM potassium phosphate (K-Pi) buffer, pH 7.6, 0.4 M NaCl, 1 mM EDTA, 0.1 M *p*-phenyl-methylsulfonyl fluoride (PMSF), 1 μ g/mL leupeptin and 1 μ g/mL pepstatin A; the NaCl concentration was changed as specified. To estimate relative yield and solubility of samples, 20 μ L of the crude extract was centrifuged at 6700 g for 10 min at 20 °C; the supernatant was subjected to SDS-PAGE using 11% acrylamide. Electrophoresis was run at 100 V for 40 min using Tris-glycine buffer at pH 8.3 (Laemmli, 1970). Twelve microliters of samples containing 3 μ g protein was applied in each well. The amount of expressed protein was estimated by scanning the SDS gel patterns and determining intensity using the Scion Image software (Scion Corporation, Maryland, U.S.A.).

2.4. Protein content determination

Protein content was determined using Bradford (1976) method with bovine serum albumin as standard.

2.5. Protein purification

Ammonium sulfate (21 g) was added to the crude protein extract (100 mL) to 35% saturation and then, subjected to hydrophobic interaction chromatography (HIC) using butyl Toyopearl. The sample was eluted with ammonium sulfate gradient of 30 to 0% saturation in buffer A (described earlier). Fractions were run on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and those that contained the unmodified and mutant 8S α globulin were collected and dialyzed against 35 mM potassium phosphate buffer, 1 mM EDTA, 1 μ g/mL pepstatin A, 1 μ g/mL leupeptin, and 0.1 mM *p*-phenyl-methylsulfonyl fluoride (PMSF) containing 0.1 M NaCl.

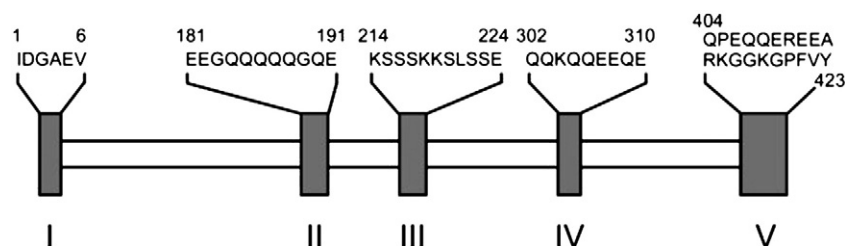


Fig. 1. Schematic diagram of disordered regions (I to V) of mungbean 8S α globulin gene based on Itoh et al. (2006).

Table 1

Wild type and modified mungbean 8Sα globulin: modified amino acid sequences and primers for PCR.

Name (No. of Met) ^a	Wild type sequence (disordered region)	Modified sequence (disordered region) ^b	PCR reaction		
			Template	Forward primer ^c	Reverse primer
Ila (3)	EEGQQQQQQQE (II)	MMM QQQQQQQE (II)	WT	5'AGGGTTCTGTTTGGAA <u>ATGATGATG</u> CAGCAACAAC3'	5'TCCAAACAGAACCC TGCTTATCTCC3'
Ila + b (5)	EEGQQQQQQQE (II)	MMM QQQQQQ MM (II)	Ila	5'CAGCAACAACAGCAA <u>ATGCAGATG</u> AGTCAGCAAG3'	5'TTGCTGTTGTTGCTG CATCATCATAG3'
IVa (2)	QQKQQEEQE (IV)	QMM QQEEQE (IV)	WT	5'GGCCAAAGAGAA <u>CAATGATG</u> CAG CAAGAAG 3'	5'TTGTTCTCTTTGGCC AACAAAGTTCAA3'
IVa + b (5)	QQKQQEEQE (IV)	QMM QQ MM (IV)	IVa	5'CAAATGATGCAGCAA <u>ATGATG</u> CA <u>ATG</u> GAAAGTTGGG 3'	5'TGCTGCATCATTTG TTCTCTCTTTG3'
II + 4 (4)	EEGQQQQQQQE (II)	EEGQQ MMMM QQQE (II)	WT	5'GAGAGGAGGGACAGCAACAA <u>ATGATGATGATG</u> CAGCAAGGCC3'	5'TGTGCTGTCCCTCC TCTCCAAACAGAAC3'
IV + 4 (4)	QQKQQEEQE (IV)	QQK MMMM QQEEQE (IV)	WT	5'AAAGAGAACACAGAAAGCAG <u>ATGATGATGATG</u> CAAGAAGAAC3'	5'CTGTTCTGTTGTTCT CTTTGGCAACAA3'
IVa + b + Ila (8)	QQKQQEEQE (IV) EEGQQQQQQQE (II)	QMM QQ MM (IV) MMM QQQQQQQE (II)	IVa + b	5'AGGGTTCTGTTTGGAA <u>ATGATGATG</u> CAGC AACAAAC3'	5'TCCAAACAGAACCC TGCTTATCTCC3'
IVa + b + Ila + b (10)	QQKQQEEQE (IV) EEGQQQQQQQE (II)	QMM QQ MM (IV) MMM QQQQ MM (II)	IVa + b + Ila	5'CAGCAACAACAGCAA <u>ATGCAGATG</u> AGCAAG3'	5'TTGCTGTTGTTGCTG CATCATCATAG3'

^a Total number of substituted/inserted Met residues is shown in parentheses.^b Bold italics refer to modified amino acids.^c Bold underlined italics refer to codons for methionine introduced by the PCR.

2.6. Analysis of trimer formation

The purified sample (0.5 mg in 1 mL) was applied on a Sephacryl S-200 HR column (1.5 cm × 25.5 cm) (Amersham Pharmacia Biotech) and eluted with buffer A at 0.4 mL/min flow rate. Fractions (1 mL) were collected and read using a SmartSpec 3000 UV/Vis Spectrophotometer (Bio-Rad) at 280 nm.

2.7. Differential scanning calorimetry (DSC) measurement

DSC analysis was carried out as described previously (Maruyama et al., 1998) using 1 mg/mL protein solution dialyzed in buffer A ($\mu=0.5$). Scanning was recorded using MicroCal MC-2 Ultra-Sensitive Micro-calorimeter (Micro Cal Inc) at a rate of 1 °C/min from 30 to 110 °C under nitrogen gas.

2.8. Emulsifying ability

Protein-stabilized oil-in-water emulsions were prepared according to the method described by Kim and Kinsella (1987) and Tandang, Atsuta, Maruyama, Adachi, and Utsumi (2005). Protein solutions (2 mg/mL) were dialyzed against 35 mM potassium phosphate buffer at 0.4 M sodium chloride concentration. For the emulsification test, the test protein solution (1.5 mL) and soybean oil (0.25 mL) were homogenized using a Heidolph Diast 900 homogenizer for 30 s at maximum rpm followed by 1 min sonication in a Branson Sonifier Cell Disruptor 185 at 100 μ A. The stability of the emulsion was observed at 0, 12 and 24 h.

2.9. Calculation of amino acid score

The amino acid scores of the WT and the mutant (IVa + b + Ila + b) were calculated according to the recommended amino acid scoring patterns for pre-school children of the FAO/WHO/UNU (1985).

2.10. Assessing allergenicity potential of the expressed protein

The prediction of allergenicity potential of the WT and mutant proteins was assessed using BLASTP of the National Center for Bioinformatics Institute (<http://www.ncbi.nlm.nih.gov/>) website (Altschul, Gish, Miller, Myers, & Lipman, 1990) and the Allergen Database for Food Safety (ADFS) (<http://allergen.nih.gov.jp/ADFS>) website (Stadler & Stadler, 2003).

3. Results and discussion

3.1. Molecular design of 8Sα globulin with engineered methionine residues

Based on the X-ray crystal structure of 8Sα globulin of mungbean (Itoh et al., 2006), five disordered regions were identified (Fig. 1), specifically, regions II and IV, as the sites to add the Met to, rather than the core regions because the disordered regions would be more tolerant to modification. This is in agreement with the results of the modeling study by Dyer, Nelson, and Murai (1993) indicating that loops and turns are most appropriate for modification using phaseolin as test protein, as well as with the results of Kim et al. (1990) on modified soybean glycinin. We did not consider the disordered regions I and V which are the N- and C-terminal regions, respectively, and disordered region III, the most stretched region connecting the N- and C-halves of the molecule based on its X-ray structure (Itoh et al., 2006). If modified, these regions may be removed by proteolysis during the accumulation of the protein in the cell.

Disordered regions II and IV are composed of mostly hydrophilic residues. Disordered region II (amino acid number 181–191) consists of the sequence EEGQQQQQQQE and the disordered region IV (302–310) consists of QQKQQEEQE (Fig. 1, Table 1). To assist in the design of the modified region, we modeled the disordered regions II and IV by using the program Loopy (Xiang et al., 2002) and calculated the electrostatic energy using the program Delphi (Rocchia et al., 2002) to determine the stability of the proposed mutations. We tested the replacement of only 5 amino acids with 5 Met residues. For disordered region II, 126 possible modified proteins were calculated. One modified protein was identified to be the most stable and which was more stable than the WT. Its modeled sequence is MMMQQQQMMQ where hydrophilic glutamic acid (E) at positions 181, 182 and 191 and glycine (G) at positions 183 and 189 were replaced by Met residues. For disordered region IV, the most stable among the possible 126 modified proteins was identified to have the sequence QMMQQMMQ, where glutamine (Q) at position 303, lysine at position 304 and glutamic acid (E) at positions 307, 308 and 310 were replaced by Met residues. These two modified proteins (Ila + b and IVa + b, respectively) have 5 Met each for a total of 10 introduced Met (Table 1). To assess the effect of insertion instead of replacement, modified proteins II + 4 and IV + 4 were designed, making the loop longer with four additional Met. On the other hand, IVa and Ila with 2 and 3 Met, respectively, were designed to investigate properties of modified proteins with a smaller number of introduced Met residues.

After determining the most stable configuration of the modified proteins, the primers for the first ten Met to be added by replacing existing amino acids and to be inserted were designed and prepared. The strategy consists of sequential replacement of the amino acid residues until the replacement and/or insertion of the target number of Met residues are attained (Table 1).

3.2. Site-directed mutagenesis and characterization of the DNA sequences

All resulting modified proteins had only one band of 6500 bp which corresponds to the length of the cDNA of mungbean vicilin 8S α subunit and the vector pET21d. Analysis of the DNA sequences of the mutants showed that the codons for the replaced amino acids were ATG and no other mutation occurred in the sequence (data not shown) thus confirming the success of the mutagenesis.

3.3. Expression of the 8S α mutants

The following had earlier been proposed to serve as criteria to judge the formation of proper conformation of recombinant proglycinins (Katsube et al., 1994; Kim et al., 1990; Utsumi et al., 1993): (1) high level of expression (higher than 10% of total *E. coli* proteins); (2) solubility should be comparable to that of the native protein; and (3) expressed proteins should be able to self-assemble to trimers. Modified proglycinins have been developed which satisfied these criteria and which were also observed to accumulate readily in the protein storage vacuole (Katsube et al., 1994; Kim et al., 1990; Utsumi et al., 1993).

The expression of the WT and the Met-rich proteins in *E. coli* HMS174 (DE3) (Fig. 2) was studied to assess if they satisfy these criteria. The molecular sizes of the expressed modified proteins were confirmed to be the same as that of the WT as expected by SDS–PAGE analysis. Using densitometric analysis, the levels of expression of the modified proteins with reference to WT of each modified 8S α globulin were 96% for IVa (2) and IIa (3), 92% for IVa + b (5) and IIa + b (5), 91% for IVa + b + IIa (8), 89% for IVa + b + IIa + b (10), and 85% for IV + 4 (4) and II + 4 (4). The production of WT was calculated to be 32% and was found to be the same as that for IVa (2), IIa (3), IVa + b (5), IIa + b (5) while the production of IVa + b + IIa (8), IVa + b + IIa + b (10), IV + 4 (4) and II + 4 (4) was calculated to be 30%. (The number of Met substituted/added or inserted is enclosed in parentheses.) Notably, WT and modified proteins with 2 and 3 Met substituted had similar high % expression and production (96% and 32%). On the other hand, II + 4 and IV + 4 which had 4 Met inserted in regions II and IV, respectively, had relatively lower expression and production (85% and 30%). The modification by insertion results in longer modified proteins and this could affect their structural stability or folding ability. The lower expression of II + 4 and IV + 4 suggests that the length of the disordered regions II and IV should not be changed for modification. The yields obtained for the Met-rich proteins are relatively higher than those previously reported for modified

glycinins, e.g., up to 20% with Met inserts (Kim et al., 1990), 14% with SH and SS changes (Adachi et al., 2004), and up to 25% with multiple peptide inserts (Prak, Maruyama, Maruyama, & Utsumi, 2006). The yields expressed in *E. coli* strains were found to vary depending on the mutation and the bacterial strain used.

The purified WT and modified 8S α globulins had SDS gel electrophoretic patterns characteristic of purified 8S α globulin (Fig. 3 inset) and were subjected to further characterization such as analysis of trimer formation, emulsifying ability, thermal stability and potential nutritional quality.

For characterization experiments, the mutants expressed in large scale were IVa, IIa, IIa + b, IVa + b and IVa + b + IIa + b with 2, 3, 5, 5 and 10 Met, respectively because they had relatively higher expression than the other modified proteins. IVa + b + IIa with 8 Met substituted was not expressed in large scale because this was used only to build up the Met content, with 10 Met as the final product.

3.4. Analysis of trimer formation

The assembly into trimers of the Met-rich modified proteins was determined by their elution on gel chromatography column at pH 7.6 and ionic strength of 0.5. The WT and Met-rich proteins eluted at the same volume on Sephacryl S-200 column (Fig. 3) indicating that the modified proteins formed stable trimers similar to the recombinant 8S α globulin and native 8S globulin of mungbean (Garcia et al., 2006; Itoh et al., 2006; Tecson-Mendoza et al., 2001). Inset (Fig. 3) shows the SDS gel bands of the purified proteins. The results confirm that the modified 8S α globulin formed a three-dimensional structure similar to that of the native 8S globulin and the recombinant 8S α globulin.

3.5. Emulsifying ability

The emulsifying ability is a functional property of a protein to stabilize emulsions with a given lipid or oil. Proteins such as globulins are used as emulsifiers or stabilizers since they contain hydrophilic and hydrophobic portions. The hydrophobic groups are removed from contact with the aqueous phase while charged groups maximize solvent contacts.

The Met-rich proteins exhibited better emulsifying ability and stability than the WT (Fig. 4), especially the 10-Met IVa + b + IIa + b which had the best emulsifying ability and emulsion stability indicating that the added Met residues have contributed to increasing the hydrophobic character of the modified proteins. Earlier studies established that an increase in the hydrophobic character of the protein stabilizes its ability to form emulsions (Kato & Nakai, 1980; Shimizu, Takahashi, Kaminogawa, & Yanauchi, 1983).

3.6. Determination of melting temperature (T_m) by differential scanning calorimetry

To analyze the thermal stability of mutants, DSC experiments were performed. If the introduced Met residues were mostly exposed to the solvent, the modified protein would show lower T_m values because of the hydrophobicity of the side chain (Radzicka & Wolfenden, 1988). However, the results showed that all of the modified proteins had 3–4 °C higher T_m than the WT, with IVa + b + IIa + b exhibiting the highest T_m (Table 2). This implies that the disordered regions are now more hydrophobic with the introduced Met residues. These hydrophobic interactions increase the stability of the protein's structure (Kato & Yutani, 1988).

3.7. Potential nutritional quality

3.7.1. Amino acid score

Following the 1985 FAO/WHO/UNU recommended amino acid score patterns for pre-school children, the amino acid score of the WT was calculated as 41% for Met. For the modified proteins IIa, IIa + b, IVa + b

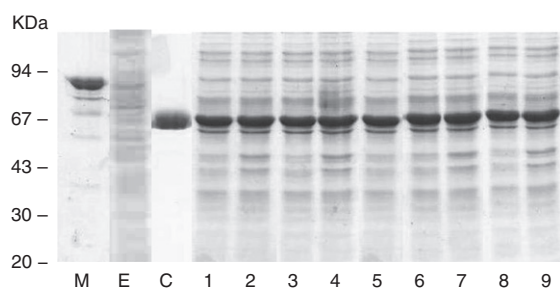


Fig. 2. SDS–PAGE analyses for expression level of WT and mutants in *E. coli* HMS 174 (DE3). (M) Molecular Weight Marker; (E) Expression profile of *E. coli* without gene insert; (C) Purified WT; 1, Crude WT; 2, IIa; 3, IVa; 4, II + 4; 5, IV + 4; 6, IIa + b; 7, IVa + b; 8, IVa + b + IIa; and 9, IVa + b + IIa + b.

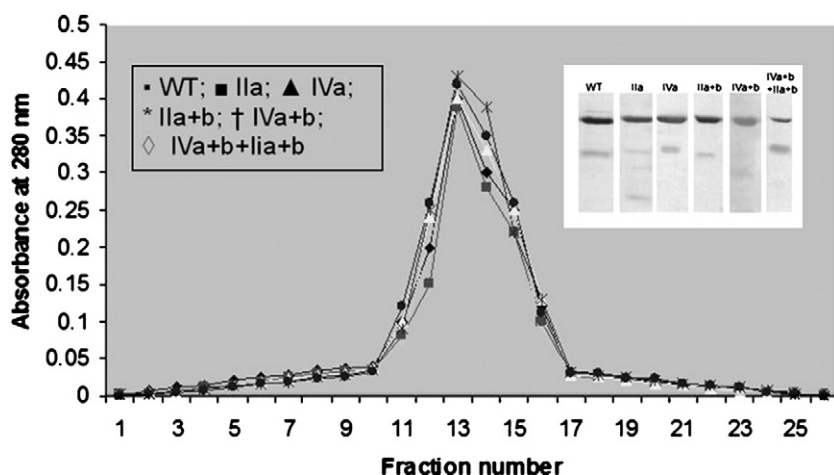


Fig. 3. Elution profile of the WT and Met-rich modified proteins on gel filtration using Sephacryl S200 HR column (1.5 cm × 15.5 cm) and buffer A at pH 7.6 and 0.4 M NaCl. Legend: smaller square – WT; larger square – IIa; triangle – IVa; asterisk – IIa + b; cross – IVa + b; and diamond – IVa + b + IIa + b. Inset, SDS gel profile of purified WT and mutant proteins: WT; IIa; IVa; IIa + b; IVa + b; and IVa + b + IIa + b.

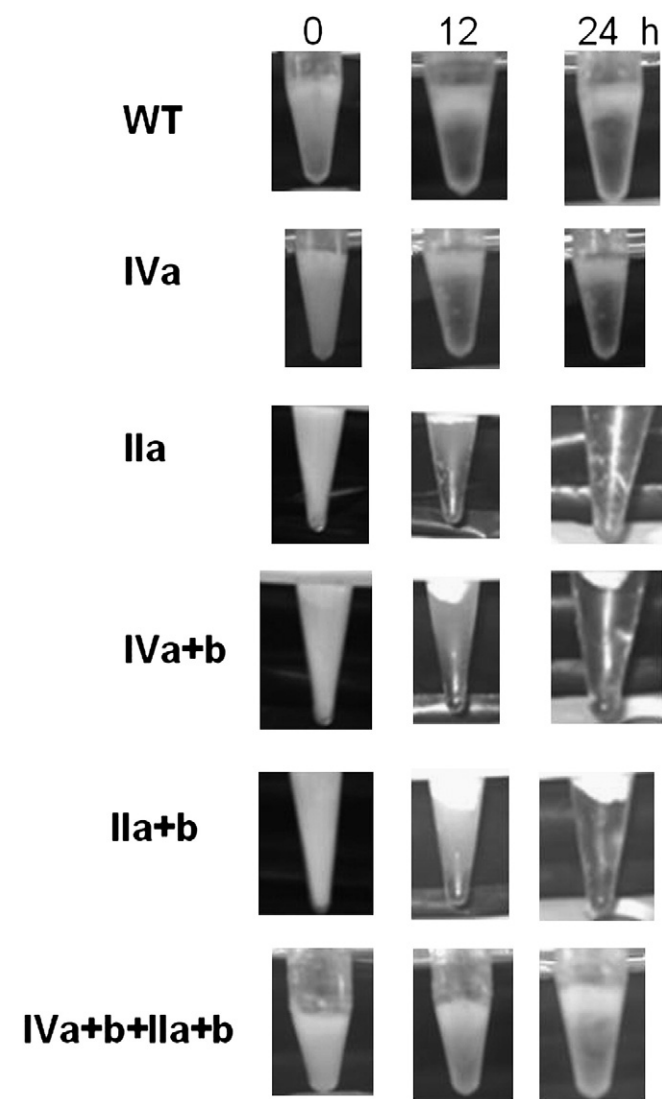


Fig. 4. Emulsifying ability of WT and the five Met-modified proteins after 0, 12 and 24 h.

and IVa + b + IIa + b, with 3, 5, 5 and 10 engineered Met plus the endogenous 4 Met per subunit, percent of number of Met residues relative to required number increased to 74, 96, 96 and 145%, indicating the potentially higher nutritional quality of the modified proteins in terms of amino acid score. The limiting amino acid changed from Met to threonine for the 5- and 10-Met rich proteins. These values are higher than the reported mungbean amino acid scores of 32% (Tsou et al., 1979) and 46% (Khalil, 2006) with the sulfur amino acids cysteine and methionine as limiting amino acids. Although the amino acid score does not give the complete picture of a protein's nutritional quality, it does provide an initial estimate. The most widely adopted method of estimating protein quality is the protein digestibility-corrected amino acid score (PDCAAS) in which the chemical or amino acid score is corrected for the true fecal digestibility of the test protein by using rat assay (FAO/WHO Expert Consultation, 1990).

3.7.2. Allergenicity potential

For new proteins in novel foods or genetically modified crops, Codex Alimentarius recommended the evaluation of new proteins initially by bioinformatic analysis (FAO & WHO, 2009). Initially, an exhaustive search in the literature regarding mungbean as an allergenic food showed only one study which identified a Bet v1 homologous protein in mungbean (and other legumes) which causes birch pollen allergic patients to suffer allergic reaction to mungbean seedlings or sprouts (Mittag et al., 2005). The Bet v1 in mungbean is not present in the seed but only in the seedling, is heat labile (Mittag et al., 2005) and belongs to the family of pathogenesis-related proteins (PR-10).

We subjected the eight Met-modified proteins to bioinformatic analysis for potential allergenicity comparing their sequences with those of known allergens as recommended by the CODEX Alimentarius guidelines (FAO & WHO, 2009) for new proteins in novel foods or

Table 2

Melting temperature (T_m) of the WT and the five modified proteins obtained by differential scanning calorimetry (DSC).

Proteins	T_m (°C)
WT	85.3
IIa	86.5
IVa	87.0
IIa + b	87.3
IVa + b	88.0
IVa + b + IIa + b	89.0

8Sα globulin (WT and mutants) Ara h 3	G F S K N I L E A G F T P E F L E
8Sα globulin (WT and mutants) Ara h 1	A K S S S - - K K S A K S S P Y R K K
8Sα globulin (WT and mutants) Blo t 10	F E I T P E K N P Q L I T P E
8Sα globulin (WT and mutants) Ara h 1	N N Q R N F L A G E H R I F L A G D
8Sα globulin (WT and mutants) Ara h 3	N N Q R N F L A G E N N Q E F

Fig. 5. Protein sequence alignment of similar amino acid sequences (from the sequence alignment between allergen Len c 1.0102 and 8Sα globulin) of 8Sα globulin (top) and the known allergen epitopes (below). Bold letters indicate identical sequence.

genetically modified crops. Based on BLASTP, all protein sequences of mutants and the WT had 55% identity (E value 2.0×10^{-111}) to allergen Len c 1.0102 (emb|CAD87731.1|), the vicilin from lentil seeds. However, when epitope analysis was conducted, most of the similar amino acid sequences with 3–5 amino acid residues had no similarity with any known IgE epitope. Moreover, longer similar amino acid sequence containing 6–11 amino acid had only 3–6 non-contiguous amino acid similarity to some IgE epitope (Fig. 5). In addition, homology analysis of the Met mutants and wild type with the Bet v1 allergen did not show 3 or longer contiguous similarity with the WT or 10-Met modified protein. Studies have shown that short amino acid sequence matches of eight amino acids or fewer provide no or little value to allergy assessments for novel proteins as they give excessive identification of false-positive matches due to random chance (Silvanovich et al., 2006). This analysis indicates that the 8Sα globulin (WT and mutants) had no biologically relevant sequence with allergenicity potential and, thus, cannot be considered an allergen.

4. Concluding remarks

This study demonstrates the successful design and production of the methionine-rich mungbean vicilins with stable conformation, greater emulsifying ability and emulsion stability, greater thermal stability, and potentially better nutritional quality. Work on the expression of the mutants in plant cells is now in progress.

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