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

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ABSTRACT

The major storage protein of mungbean, 8Sα 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 8Sα 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.

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-βconglycinin. Further study revealed that mungbean vicilin has three isoforms, 8Sα, 8Sα′ and 8Sβ (Bernardo et al., 2004). The 8Sα 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 8Sα globulin of mungbean was established for the first time by X-ray crystallography (Itoh et al.,

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[2] This paper is dedicated to the memory of Professor Shigeru Utsumi who passed away on 1 December 2008.

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2006). The refined 8Sα 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 8Sα 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 8Sα 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 8Sα 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 8Sα 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 8Sα 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 8Sα subunit and pET-21d vector was used for transfection into *E. coli* DH5α 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 μg/mL carbenicillin and incubated at 37 °C until OD_{600} 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 μg/mL leupeptin and 1 μg/mL pepstatin A; the NaCl concentration was changed as specified. To estimate relative yield and solubility of samples, 20 μL 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 μg 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 8Sα globulin were collected and dialyzed against 35 mM potassium phosphate buffer, 1 mM EDTA, 1 μg/mL pepstatin A, 1 μg/mL leupeptin, and 0.1 mM *p*-phenyl-methylsulfonyl fluoride (PMSF) containing 0.1 M NaCl.

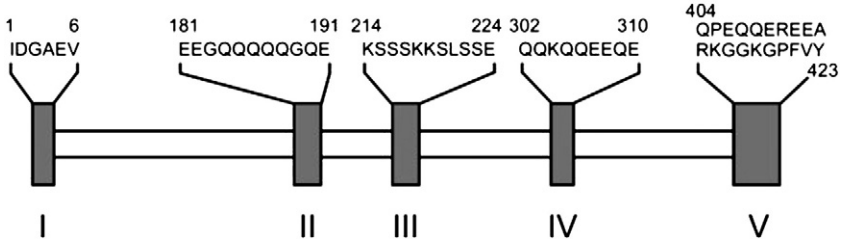

Fig. 1. Schematic diagram of disordered regions (I to V) of mungbean 8Sα globulin gene based on Itoh et al. (2006).