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Modification of Solubility and Heat-Induced Gelation of Amaranth 11S Globulin by Protein Engineering

Laura Carrazco-Peña,[†,‡] Juan A. Osuna-Castro,[*,§] Antonio De León-Rodríguez,[‡] Nobuyuki Maruyama,[∥] Jorge F. Toro-Vazquez,[⊥] Juan A. Morales-Rueda,[⊥] and Ana P. Barba de la Rosa[*,‡]

[†]Facultad de Ciencias Químicas, Universidad de Colima, Carr. Colima−Coquimatlán, km 9, 28400, Coquimatlán, Colima CP 28400, Mexico

[‡]IPICyT, Instituto Potosino de Investigación Científica y Tecnológica A.C. Camino a la Presa San José No. 2055, Lomas 4a sección, 78216 San Luis Potosí, S.L.P., Mexico

[§]Facultad de Ciencias Biológicas y Agropecuarias, Universidad de Colima, km 40 Autopista Colima-Manzanillo, 28100 Tecomán, Colima, Mexico

[∥]Laboratory of Food Quality Design and Development, Graduate School of Agriculture, Kyoto University, Uji, Kyoto 611-0011, Japan

[⊥]Facultad de Ciencias Químicas—CIEP, Universidad Autónoma de San Luis Potosí, San Luis Potosí, S.L.P., Mexico

Supporting Information

ABSTRACT: The primary structure of amaranth 11S globulin (*Ah*11S) was engineered with the aim to improve its functional properties. Four continuous methionines were inserted in variable region V, obtaining the *Ah*11Sr+4M construction. Changes on protein structure and surface characteristics were analyzed in silico. Solubility and heat-induced gelation of recombinant amaranth 11S proglobulin (*Ah*11Sr and *Ah*11Sr+4M) were compared with the native protein (*Ah*11Sn) purified from amaranth seed flour. The *Ah*11Sr+4 M showed the highest surface hydrophobicity, but as consequence the solubility was reduced. At low ionic strength (μ = 0.2) and acidic pH (<4.1), the recombinant proteins *Ah*11Sr and *Ah*11Sr+4 M had the highest and lowest solubility values, respectively. All globulins samples formed gels at 90 °C and low ionic strength, but *Ah*11Sn produced the weakest and *Ah*11Sr the strongest gels. Differential scanning calorimetry analysis under gel forming conditions revealed only exothermic transitions for all amaranth 11S globulins analyzed. In conclusion, the 3D structure analysis has revealed interesting molecular features that could explain the thermal resistance and gel forming ability of amaranth 11S globulins. The incorporation of four continuous methionines in amaranth increased the hydrophobicity, and self-supporting gels formed had intermediate hardness between *Ah*11Sn and *Ah*11Sr. These functional properties could be used in the food industry for the development of new products based on amaranth proteins.

KEYWORDS: *Amaranthus hypochondriacus 11S globulins, molecular modeling, recombinant proteins, site directed mutagenesis, thermal-mechanical properties*

INTRODUCTION

Amaranth is considered as a promising alternative crop due to its agronomical and nutritional characteristics. The plant is naturally tolerant to several abiotic stresses such as high temperatures, salinity, and water deficit.[1−3] Besides the superior nutritional quality of amaranth seed proteins that is mainly due to their well-balanced amino acid composition,[4] amaranth proteins are also rich in active peptides with several biological functions.[5−7] Amaranth is mainly consumed as candies made with popped seeds mixed with sugar cane molasses, but its use is increased worldwide as ground flour that is blended with wheat or other flours to make cereals, crackers, cookies, bread, or other baked products.[8]

The uses of proteins in food systems depends mainly on their physicochemical and functional properties.[9] Solubility, heat coagulation, thermal stability, emulsifying activity, foaming capacity and stability, and gelation properties of amaranth protein isolates have been investigated.[10−12] However, the physicochemical and functional properties of pure amaranth storage proteins are not well-known. This information is of utmost importance to support the use of individual amaranth proteins in commercial food products. Additionally, it might also contribute to the design strategies for the rational improvement of their functionality.[13]

The 11S globulins (salt-soluble proteins) are one of the major storage protein fractions in amaranth.[4,14] Amaranth 11S globulin has a molecular mass around 398 kDa, and under reduced conditions it shows characteristic bands of the acidic (36−32 kDa) and basic subunits (24−22 kDa) of 11S proteins.[15] Additionally, a 59−55 kDa band corresponding to the nonprocessed proglobulin suggests that this protein is synthesized as a precursor and has structural characteristics similar to other 11S globulins.[15] The 11S globulin subunits are synthesized as a single polypeptide precursor as a preproprotein, from which the signal sequence is separated cotranslationally.[16] The resultant proproteins assemble into trimers in the endoplasmic reticulum and transported to protein storage

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vacuoles where they are cleaved to form acidic and basic polypeptides linked by a disulfide bond.[16,17] After this cleavage, the trimers assemble into stable mature hexamers.[17]

Protein engineering tools have been used to improve the nutritional and functional properties of seed storage proteins. Modifications of the primary structure of these proteins have been done using a rational design based on structural information.[13,18] Modified proteins have been successfully expressed in bacteria such as *Escherichia coli*, allowing the analysis of functional properties.[18] Thus, the solubility of proglycinin (soybean 11S proglobulin) and cruciferin (rapeseed 11S proglobulin) has been enhanced,[19] and heat-induced gelation of proglycinin has been improved.[20] Basically, five variable regions (named I to V), identified through alignment of various legumes 11S globulins,[21] have been modified without altering the overall protein three-dimensional structures; from these regions, the number V has been the most useful for modification using methionine residues[13,18] Recently, the 3D structure of amaranth 11S proglobulin was resolved at a 2.28 Å resolution,[22] opening the possibility to investigate the structure−function relationships of this amaranth storage protein.

The aim of the present research was to apply the protein engineering tools to modify the functional properties of 11S amaranth globulin. To achieve this objective, four continuous methionines were inserted in the variable region V. The unmodified recombinant (*Ah*11Sr) and modified 11S amaranth (*Ah*11Sr+4M) proglobulin genes were overexpressed in *E. coli*. To provide information on molecular features that influences the amaranth 11S globulins functional properties, the protein surface hydrophobicity−hydrophilicity was analyzed in silico. Solubility as a function of pH was determined for native and recombinant proteins. Heat-induced gelation was analyzed at high protein concentration and low ionic strength, and under these conditions thermal-mechanical properties were determined.

MATERIALS AND METHODS

Construction of Expression Plasmid for Modified Amaranth 11S Globulin. *Amaranth hypochondriacus* 11S proglobulin cDNA (*Ah*11S) was cloned onto a pET21d expression vector.[23] The modified 11S proglobulin gene was constructed by insertion of four continuous methionines in variable region V (Figure 1) between Gly_{454} and Gln_{455} and was named as *Ah*11Sr+4M. The specific oligonucleotides used for methiones insertion were: 5′-ATGGAAGGAAGGTTTAGAGAG-3′ and 5′-TTAGGCAATGCTGATTTTCCTTCGGTACTCCCCTTG-CATCATCATCATGCCACTAGAGCGGAAAAGGG-3′, and the methionine codons are underlined. Platinum Taq DNA polymerase (Invitrogen-Life Technologies, NY, USA) was used. PCR reaction was carried out as follows: denaturation at 94 °C for 1 min; 30 cycles of 94 °C for 3s, 60 °C for 15s, 72 °C for 1.5 min. The amplified fragment was cloned onto pCR 4-TOPO (Invitrogen) and subsequently subcloned into pET21d expression plasmid (Novagen, Merck-Millipore, Darmstadt, Germany).

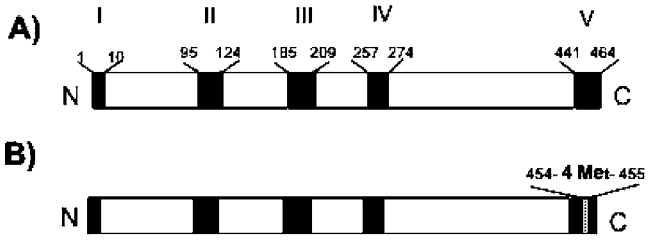

Figure 1. Schematic representation of (A) variable and conserved regions in amaranth 11S proglobulin and (B) construction of the modified proglobulins (*Ah*11Sr+4M) with the insertion of four methionines in the region V (dashed area). Open and closed areas represent conserved and variable regions, respectively.

Expression of Recombinant Amaranth 11S Globulins. *E. coli* Rosetta gami (DE3) competent cells were transformed with pET21-*Ah*11S and pET21-*Ah*11S+4M constructions. Expression conditions for pET21-*Ah*11S were described previously.[23] Expression of recombinant *Ah*11S+4M was carried out using Luria Broth (LB) containing 0.17 M NaCl and the LB modified containing 0.5 M NaCl. Flasks of 2 L containing 500 mL of medium supplemented with ampicillin (34 μg/mL) were inoculated with 2 mL of overnight cell suspension. The cultures were incubated at 37 °C with constant agitation at 180 rpm until OD_{600} of about 0.6. At this point, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. The induced cultures were incubated at 30 °C with constant agitation at 90 rpm. Aliquots were taken before induction and 40 h after induction. The cells were harvested by centrifugation and resuspended in buffer A (35 mM potassium phosphate pH 7.6, 0.1 M NaCl, 1 mM EDTA, 10 mM 2-mercaptoethanol) containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF). The total, soluble, and insoluble proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 12% gel and stained with colloidal Coomassie blue.[24]

Purification of Native 11S Globulin from Amaranth Seeds. The native 11S globulins (*Ah*11Sn) were extracted and purified from mature amaranth (*Amaranthus hypochondriacus* L. cv. Gabriela) seeds. Seeds were ground to a fine flour (100 mesh) that was defatted with hexane.[4] Suspensions of flour:water 1:10 (w/v) were stirred for 30 min at 4 °C, and albumins fraction was removed by centrifugation at 11000*g* for 30 min. The pellet was resuspended in buffer B (0.1 M NaCl, 10 mM K_2HPO_4, 1 mM EDTA pH 7.6), stirred and centrifuged. The insoluble fraction (pellet) was dissolved in buffer C (0.4 M NaCl, 10 mM K_2HPO_4, 1 mM EDTA, pH 7.6), stirred, and centrifuged. The resulting supernatant (20 mL) containing mainly the native 11S globulin was dialyzed against 2 L of distilled water with three changes of dialysis water. The sample was lyophilized and resuspended in buffer C. Further purification of 11S globulins was done through a gel filtration column as described below.

Purification of Recombinant Amaranth 11S Proglobulins. *E. coli* cells transformed with pET21-*Ah*11S or pET21-*Ah*11+4M were resuspended in buffer A and lysed by sonication. Soluble proteins were released, recovered by centrifugation at 11000*g* for 30 min at 4 °C, and subjected to ammonium sulfate (AS) fractionation.[25] For purification of recombinant 11S proglobulin (*Ah*11Sr), the protein was fractionated at 40% AS saturation. Precipitated protein was collected by centrifugation and dissolved in buffer D (35 mM potassium phosphate pH 7.6, 0.4 M NaCl, 1 mM EDTA, 10 mM 2-mercaptoethanol, 0.1 mM PMSF) and dialyzed against 12 L of buffer A at 4 °C for 18 h. The recombinant protein was recovered by centrifugation and resuspended in buffer D. For purification of modified recombinant 11S proglobulin V+4M (*Ah*11Sr+4M), the *E. coli* total soluble proteins were fractionated with precipitation at 30% of AS saturation. The precipitate was resuspended in 8 mL of buffer C, clarified by centrifugation, and applied on a 2.5 cm × 68 cm Sephacryl S-300 column (GE Healthcare, Piscataway, NJ, USA) using buffer C as a mobile phase at a flow rate of 0.5 mL/min at room temperature. The void volume of the column and elution volume of protein standards were determined as reference. Fractions of 4 mL were collected and analyzed by SDS-PAGE.

Protein Modeling of Proglobulins *Ah*11Sr and *Ah*11Sr+4M. The 3D model of *Ah*11Sr+4 M was obtained using the Swiss-PDB Viewer 4.04[26] and the ModLoop[27] found at http://modbase.compbio.ucsf.edu/modloop/. The 3D structure of unmodified recombinant amaranth 11S globulin with PDB access 3QAC[22] was used as a template. The variables regions II and IV were added with Scan Loop database and regions I, III, and V with ModLoop web server (http://modbase.compbio.ucsf.edu/modloop/). Energy minimization was carried out with GROMOS96 (Swiss-Pdb Viewer 4.04 software) 200

cycles of steepest descent to correct the stereochemistry of the model.[26] For quality evaluation, the model was submitted to PSVS[28] (http://psvs-1_4-dev.nesg.org/) using *Z* score computed by Prosa II,[29] *G* factor values and Ramachandran $\varphi-\psi$ plots calculated with PROCHECK.[30] The models of the loops added to the 11S amaranth globulin were checked by Verify 3D (http://nihserver.mbi.ucla.edu/Verify3D/).[31] Structures were visualized using Swiss-PDB Viewer 4.04.[26]

Analysis of Surface Properties of Native and Recombinant Amaranth Proglobulin. Hydrophobic, hydrophilic, and electrostatic potential on the molecular surface of 11S proglobulins were calculated and visualized using the Swiss-Pdb Viewer 4.04 software. The electrostatic potentials of the proteins were computed with the Poisson−Boltzmann method implemented in the program. The dielectric constant of 2 and 80 for protein and solvent, respectively, were used at ionic strength (μ) of 0.2.

Protein Solubility As a Function of pH. The solubility of different 11S globulins was determined as described by Lakemond et al.[32] Briefly, the pH of different protein solutions (0.8 mg/mL) on buffer A (low ionic strength, μ = 0.2) or C (high ionic strength, μ = 0.5) was modified by adding solutions of HCl (0.05−5 M) with constant stirring until achieving a particular pH between 2.6 and 7.6 at 0.5 pH unit intervals. The solutions were incubated 20 h at 20 °C and then centrifuged (11000*g* for 15 min). The concentration of soluble protein was determined using the Protein Assay Kit (Bio-Rad, Hercules, CA, USA) with BSA as the standard protein.

Heat-Induced Gelation by Oscillatory Rheology. The gelation of 11S globulins solutions was evaluated by oscillatory rheological measurements using a MCR 301 rheometer (Anton Paars, Stuttgard, Germany) equipped with a true-gap system with a cone and plate geometry (1° and 25 mm diameter CP 25−1/TG). Different concentrations of protein solutions were tested (7, 15, 20, and 21.8%). Sample was applied on the base of the plate, and the cone was set using the true-gap function of the rheometer software. The protein solution was heated for 20 min at 90 °C and then cooled (10 °C/min) until 20 °C. Then a frequency sweep between 10 and 0.01 Hz was applied using a strain of 0.1%. These conditions assured rheological measurements within the linear viscoelastic region (LVR) of the system. The elastic (G') and loss (G'') modulus of the gel were obtained from the LVR of the frequency sweep. The sample temperature of the plate was controlled through a Peltier (P-PTD 200TG) and the Peltier-controlled hood (H-PTD 200). The control of the equipment was made through the equipment software (Start Rheoplus US200/32 version 2.65, Anton Paar, Graz, Austria).

Differential Scanning Calorimetry (DSC) Analysis. DSC analysis was conducted using the protein solution (tested in heat-induced gelation) in 35 mM potassium phosphate (pH 7.6). Scanning was recorded using Q1000 differential scanning calorimeter (TA Instruments, Inc., New Castle, DE, USA). An aliquot of 25 μL of each protein solution was weighed in aluminum pans and hermetically sealed. After equilibrium of the sample pan at 20 °C in the calorimeter, the system was heated at a rate of 10 °C/min until achieving 180 °C, recording the heat flux as a function of temperature. The transition temperature (T) and the enthalpy for the corresponding protein transition (ΔH, J/g) were calculated with the equipment software (TA Universal Analysis 2000 V 4.2E).

Statistical Analysis. All analyses were done at least in triplicate and data reported as mean ± standard deviation. When appropriate, data were analyzed for statistical significance ($p \leq 0.05$) using STATISTICA v7.1 program (StatSoft, Inc., Oklahoma, USA). Duncan Test was used to determine the significant differences among samples.

RESULTS AND DISCUSSION

Expression and Detection of Recombinant Amaranth 11S Proglobulins. Recombinant *Ah*11Sr and *Ah*11Sr+4M amaranth proglobulins were expressed in *E. coli* Rosetta gami (DE3). The expression and accumulation of recombinant proteins in the soluble fraction of cellular extract were increased when modified LB containing 0.5 M NaCl and temperature postinduction was maintained at 30 °C for 40 h. Under these conditions, more than 50% of the recombinant proteins were accumulated in the soluble fraction (Figure 2).

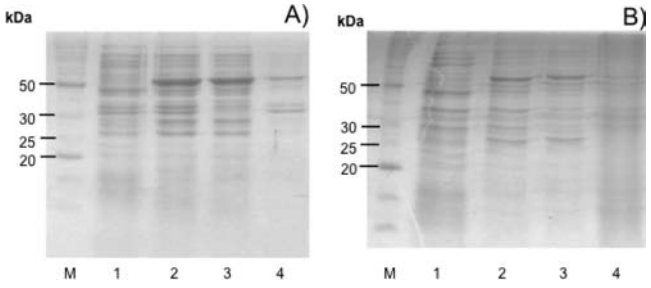

Figure 2. SDS-PAGE profile of the expression of recombinant amaranth 11S globulin. (A) *Ah*11Sr and (B) *Ah*11Sr+4M. Expression was carried out at 30 °C for 40 h. Line M = molecular weight marker, line 1 = noninduced, line 2 = induced, line 3 = soluble fraction, line 4 = insoluble fraction. The volume of 5 μL (~5 μg protein) of each sample were applied in each line.

Purification of Recombinant Amaranth 11S Proglobulins. After precipitation with AS, recombinant proteins were easily purified due its tendency to aggregation at low temperature in low ionic strength buffer. This behavior has been reported previously.[23] Purity of proteins, as determined by densitometry analysis (Figure 3), was around 95% for 11S

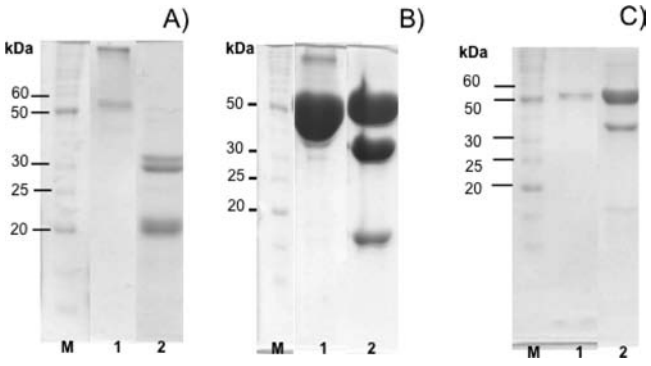

Figure 3. SDS-PAGE profile of purified 11S amaranth proglobulins. (A) *Ah*11Sn purified from amaranth seed flour, (B) *Ah*11Sr recombinant proglobulin, (C) *Ah*11Sr+4 M recombinant protein with four methionines inserted in variable region V. Line M = molecular weight marker, line 1 = nonreducing conditions, line 2 = under reducing conditions. Sample 4 μg (*Ah*11Sn), 65 μg (*Ah*11Sr), and 5 μg (*Ah*11Sr+4M) were applied.

globulins obtained from amaranth seeds (*Ah*11Sn) and approximately 90% for the both recombinant proteins (*Ah*11Sr and *Ah*11Sr+4M). Under nonreducing conditions, all proteins showed similar patterns and a main band around 54 kDa corresponding to the native proglobulin was observed (Figure 3). After sample reduction, the acidic (40−35 kDa) and basic (25−20 kDa) polypeptides were released in *Ah*11Sn (Figure 3A), but as expected in *E. coli* recombinant proteins, the posttranslational modifications are not carried out and the bands observed could be due to limited to natural proteolysis during purification steps (Figure 3B,C) as reported for modified proglycinins.[20,33]

Structural Analysis of Amaranth 11S Proglobulins. The crystal structure of *Ah*11Sr expressed in *E. coli* was resolved at 2.28 Å,[22] but in this model the variable regions were not

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observed. These variable regions are important due to their biological and food technology properties.[13,34] Using bioinformatics tools, we have built the structure models of *Ah*11Sr and *Ah*11Sr+4M including the variable regions (Figure 4). The

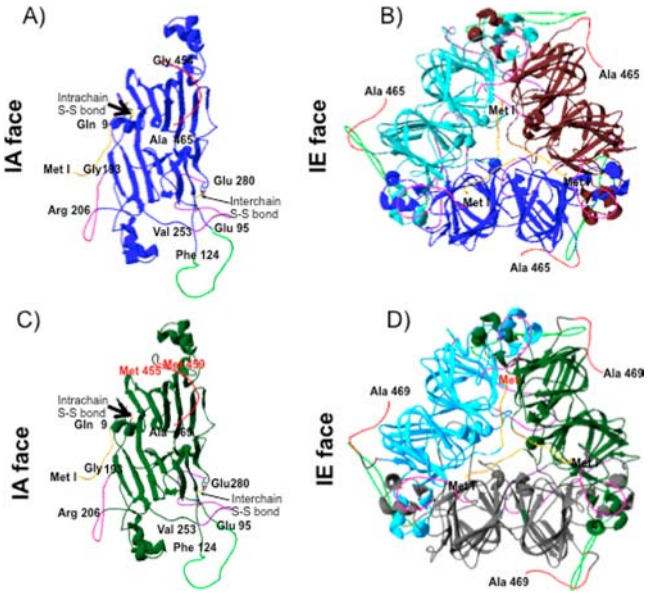

Figure 4. 3D models of recombinant amaranth 11S globulins. (A) and (B) monomer and trimer structure of *Ah*11Sr; (C) and (D) monomer and trimer structure of *Ah*11Sr+4M, respectively. Variable regions are shown in different colors. Variable region: (I) yellow, (II) green, (III) pink, (IV) violet, and (V) red. Also Met and Ala at N- and C-terminal are indicated. In the case of *Ah*11Sr+4M, the four inserted methionines are displayed in red. Intra- and interchain disulfide bonds are shown with black arrows. Trimers are IA (intrachain disulfide-containing) faces.

structure quality value, estimated by *Z*-score, was −1.57 for *Ah*11Sr and −1.65 for *Ah*11Sr+4M, (Supporting Information Tables 1 and 2). These values are considered within the defined region for *Z*-scores of structures solved by X-ray crystallography or NMR. Ramachandran plots by PROCHECK showed that the residues in the most favored and allowed regions were 98.5% for *Ah*11Sr and 99.2% for *Ah*11Sr+4M (Supporting Information Tables 1 and 2). Factor *G* calculated by PROCHECK, as an indicative of the correct stereochemistry of a given structure, was −0.38 for *Ah*11Sr and −0.37 for *Ah*11Sr+4M. These are values within the acceptable range that is reported from 0 to −0.5.[29] Verify3D evaluates the compatibility of the tridimensional model of a protein with its primary sequence, considering acceptable values >0. The profiles obtained by Verify3D were in general from 0 to 0.74 for *Ah*11Sr and 0 to 0.71 for *Ah*11Sr+4M.

As reported previously for proglycinin,[35] the variable regions I, III, and V in *Ah*11Sr and *Ah*11Sr+4 M were oriented to IA (intrachain disulfide-containing) face and regions II and IV were positioned to the IE (interchain disulfide-containing) face (Figure 4). Mature 11S globulins assemble as hexamers trough IE face after proteolytic processing in the Asn-Gly site in variable region IV.[34] The IA face remains on the hexamer surface and consequently regions I, III, and V and some parts of regions II and IV which are on the sides of the each trimer. Therefore, regions I, III, and V and exposed parts of regions II and IV would contribute to the molecule surface behavior. These observations have been useful to define that variable region V are the target for modification of 11S globulins.[13,20]

Analysis of Surface Properties of Amaranth Proglobulins. The molecule interaction capacity is an important part of protein functionality, and then the elucidation of the functional properties based on the structure of proteins is an important task in food protein research.[19,36] Attempts to relate the hydrophobic and hydrophilic amino acid content of proteins to their physical properties have been made a long time ago. However, the hydrophobicity and the charge distribution of the protein surface are the most important parameters that describe its functionality.[36]

Amaranth globulin hydrophobicity has been studied a long time ago. Konishi and Yoshimito (1989)[37] have reported that amaranth 11S globulins have the highest surface hydrophobicity (15800) among other tested proteins including soybean isolate (9600). With the recent report of the 3D structure of amaranth 11S globulin,[22] it is now possible to get more insights about the globulins structure−function relationships. As shown in Figure 5A, the distribution of aromatic hydrophobic residues on *Ah*11Sr was mainly observed on the central part of IA face. The IE face had a more extended distribution of hydrophobic residues on the molecule surface. The *Ah*11Sr+4M proglobulin had higher surface hydrophobicity as observed on its IA face, this could be attributed to the addition of four continuous methionines in variable region V which is exposed to IA face of the trimer (Figure 5C). The surface electrostatic map showed that *Ah*11Sr proglobulin is mainly negatively charged on its IA face and positive on its IE face (Figure 5B). The inclusion of variable regions into the 11S amaranth globulin structure does not significantly modify the distribution of surface charge on the molecule, nor does the addition of four continuous methionines in variable region V (Figure 5D). This balance of hydrophobic and hydrophilic surfaces in amaranth 11S could explain now the high experimental hydrophobicity as reported before.[22]

Protein Solubility Evaluation. Surface hydrophobicity has the greatest influence on the physical properties such as solubility. Solubility is an important physicochemical characteristic generally associated with functional properties such as gelation, foaming capacity, and emulsification.[38] Protein solubility of *Ah*11Sn, *Ah*11Sr, and *Ah*11Sr+4M was analyzed at 20 °C as a function of pH (7.6 to 2.6) and at two ionic strengths (μ = 0.5 and 0.2). At high ionic strength (0.5), the highest protein solubility (>80%) of all amaranth 11S globulins samples was observed in the interval of pH from 7.6 to 6.1 (Figure 6A). A this pH interval, 11S globulins from other sources are almost 100% soluble.[20,32,39] At pH lower than 6.1, the 11S amaranth globulins showed a drastic decrease in solubility (Figure 6A), reaching the lowest values of ∼10% to 20% at pH < 5.1. This pattern was also observed for other proteins such as cruciferin and proglycinin.[20,32,39]

The solubility profiles at low ionic strength (μ = 0.2) were also similar to the other 11S globulin.[20,32,39] The maximum protein solubility for amaranth globulin (>80%) was achieved from pH 7.6 to 7.1 (Figure 6B). Recombinant globulin (*Ah*11Sr, and *Ah*11Sr+4M) had the lowest solubility in the pH interval between 6.1 and 4.1, while *Ah*11Sn showed its lowest solubility between pH 5.1 and 4.1. In the three amaranth 11S globulin samples, the protein solubility increased gradually below pH 4.1. At acidic pH, the maximum solubility was observed for *Ah*11Sr (∼60%). The differences in the solubility profiles of the 11S globulin at μ = 0.2 was more evident because

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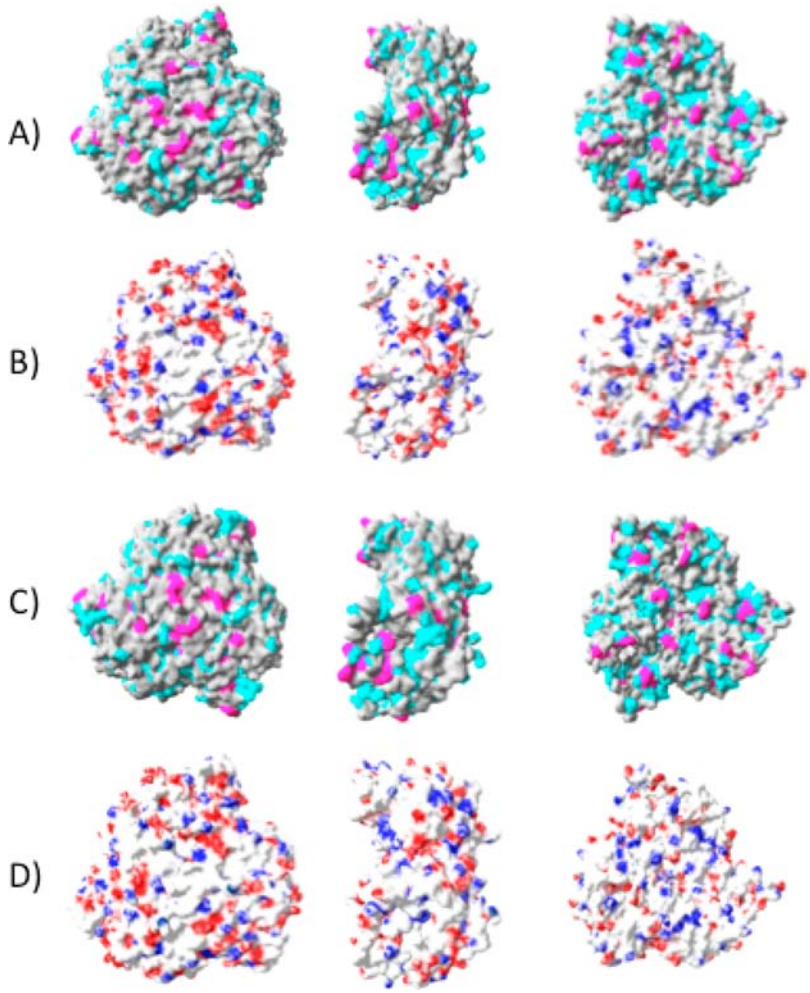

Figure 5. Surface hydrophobicity and electrostatic potential maps of recombinant amaranth 11S globulins. (A and C) Surface hydrophobicity of *Ah*11Sr and *Ah*11Sr+4M, aromatic (Phe, Tyr and Trp), and aliphatic (Ala, Gly, Ile, Leu, Met, Pro, and Val) hydrophobic residues are shown in pink and cyan, respectively. (B and D) Electrostatic potential of *Ah*11Sr and *Ah*11Sr+4M, molecular surfaces of the proteins are drawn rendering electrostatic potential from 1.8 (electropositive, red) to 1.8 Kt/e (electronegative, blue). In the left are views of IA face and in the central and right part 90 and 180° (IE face) rotations around the vertical axis, respectively, of the IA face.

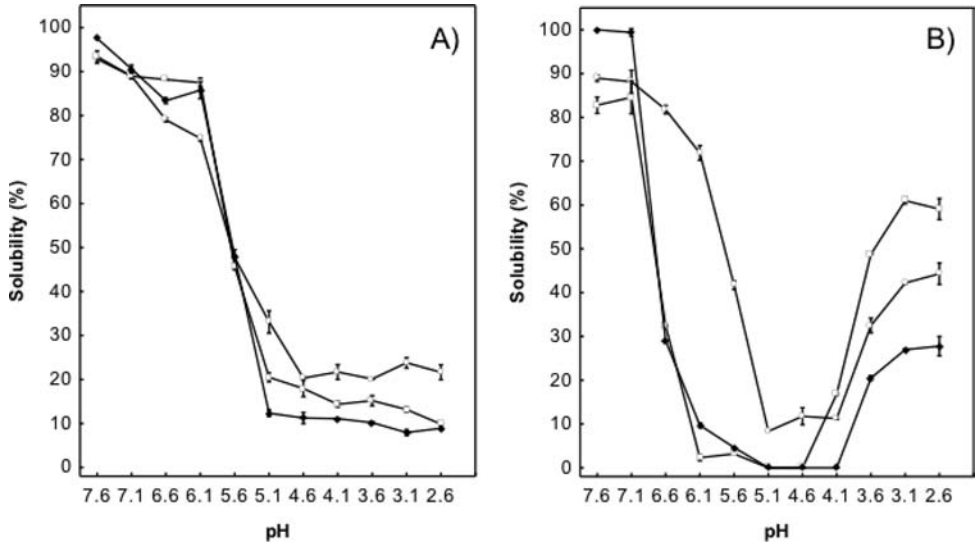

Figure 6. Protein solubility at different pH of native and recombinant amaranth 11S proglobulins measured at two different ionic strengths: (A) at high ionic strength of (μ = 0.5) and (B) low ionic strength (μ = 0.2). (○) *Ah*11Sn; (□) *Ah*11Sr, and (◆) *Ah*11Sr+4M. Vertical bars represent ± SD.

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the charge changes associated with the pH variation are not counteracted by salt ions, then changes in pH and ionic strength can alter the protein conformation exposing or hiding water binding sites.[20,32,39]

In the case of 11S globulin, the hexamer can dissociate into its constituents trimers, resulting in the probable change of surface behavior.[13,28,31] Then, the hydrophobic regions that interact in the formation of the hexamer trough IE face of each trimer are exposed, in consequence surface hydrophobicity might increase. Additionally, those regions that arise after proteolytic processing in the Asn-Gly could contribute to the change in the surface properties. The variable region IV in amaranth 11S globulin has a considerable hydrophobic distribution and thus contributes to the increase in surface hydrophobicity; this might explain that *Ah*11Sn is less soluble than *Ah*11Sr. The addition of the four continuous methionines in variable region V on *Ah*11Sr+4 M (situated on the exterior of the molecule) increased surface hydrophobicity, and as a result its solubility was lower than *Ah*11Sn and *Ah*11Sr.

Gelation Capacity Evaluated through Oscillatory Rheology. Gelation capacity is an important functional property with extensive application in food products. Heat induced gelation of globular proteins is the result of three stages: thermal denaturation of the protein, soluble aggregate formation, and their interaction to form a network which entraps water and other components.[40] The *Ah*11Sr and *Ah*11Sr+4M gelation capacity was evaluated by dynamic rheological measurements at 90 °C, at very low ionic strength (μ = 0.08) at pH 7.6. Previous reports have indicated that an amaranth protein isolate composed of albumins and globulins formed self-supporting gels from 7% concentration at 70 °C.[10,11] Initially, preliminary assays were conducted with native 11S globulin at 7%, 10%, and 15% solutions, but under these conditions the frequency sweeps G'' was always higher than G', indicating no gel formation. Figure 7 shows the gel point (G', white symbols; G'', dark symbols) values as a function of frequency sweep for each amaranth 11S proglobulin sample at 21.8% protein solution. *Ah*11Sn (lower curves) had small G' values (~5 Pa), but higher than G'', this behavior is clearly associated with a weak gel.[41] On the other hand, *Ah*11Sr had G' values from 9000 to 19000 Pa and *Ah*11Sr+4M from 130 to 300 Pa; in both samples, G' was higher than G'' over the whole frequency interval analyzed. This behavior and the rheological values are associated with strong gels.[41] Protein denaturation has been established as a requisite for heat induced gelation, however there are studies reporting only partial denaturation for gelation.[42] In the case of sunflower globulins, the gelation occurs with a thermal treatment of 20 °C, lower than its T_d.[42] Then, we have analyzed the thermal properties of amaranth globulin samples.

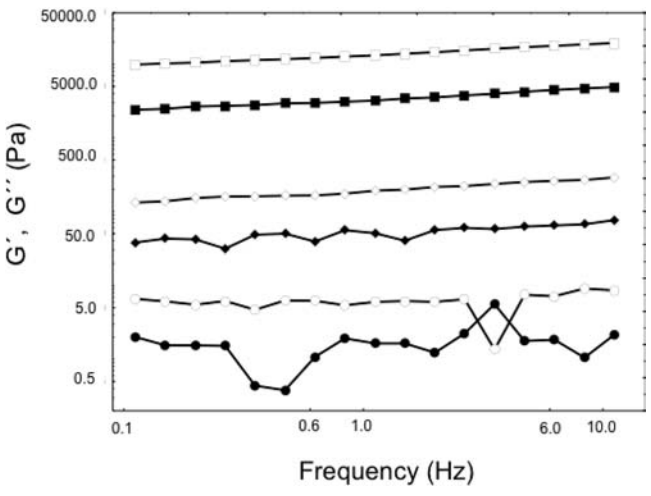

Figure 7. Frequency sweeps (G' and G'' vs frequency) of native and recombinant amaranth 11S globulin. (○, ●) *Ah*11Sn, (□, ■) *Ah*11Sr, and (◇, ◆) *Ah*11Sr+4M. G' are represented in white symbols (□, ◇, ○) and G'' in black symbols (■, ◆, ●).

Differential Scanning Calorimetry (DSC) Measurement. Amaranth 11S globulins were analyzed at the concentration used for gelation capacity (21.8%). As shown in Figure 8, the endothermic transition or protein denaturation peak as observed in amaranth and soybean protein isolates was not observed. In soybean protein isolate containing 11S and 7S globulins, only endotherms with T_d at 93.3 and 76.5 °C, respectively, have been reported.[43] At the conditions used for amaranth gel formation (high protein concentration, high temperature, low ionic strength), only the exothermic transition was observed. Exothermic events have been associated with protein aggregation, thus, the thermal parameters of the exotherm, T_{ag} and ΔH are associated with the aggregation and subsequent gelation of amaranth 11S proteins.[40] The lower aggregation temperature (T_{ag}) of 96.44 °C was observed for *Ah*11Sn and the highest for *Ah*11Sr (Figure 7, Table 1). The lowest values of ΔH or energy of aggregation was observed for *Ah*11Sn, this could be due to the fact that native proteins

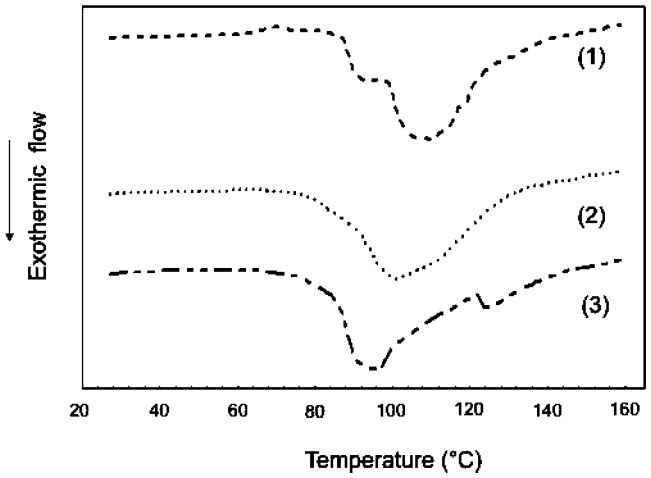

Figure 8. DSC thermograms of amaranth 11S globulins at 21.8% at low ionic strength (μ = 0.08). Curves: (1) *Ah*11Sr, (2) *Ah*11S+4M, and (3) *Ah*11Sn.

Table 1. Exothermic Transition Temperatures and Calorimetric Enthalpies of Amaranth 11S Globulins[a]

amaranth 11S globulin	T_{ag} (°C)	ΔH (J/g)
*Ah*11Sn	96.44 ± 1.35a	29.61 ± 2.86a
*Ah*11Sr	108.24 ± 2.21b	50.37 ± 3.10b
*Ah*11Sr+4M	100.24 ± 1.38a	57.18 ± 2.53b

[a] T_{ag} = temperature of aggregation, ΔH = enthalpy of transition for protein aggregation. Amaranth globulins protein solutions at 218 mg/mL were in 35 mM potassium phosphate buffer pH 7.6. Values given are means ± standard deviations. Means with different letters within a same column are significant different at $P < 0.05$.

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extracted from seeds are already processed into the basic and acidic subunits (Figure 3A) that could help for protein denaturation as a prerequisite for gel formation.[44] The ability of *Ah*11S proteins to form a gel on heating at low ionic strength could be very useful to improve the texture of food products.

There are few studies reporting exothermic transitions in proteins. It has been shown that the displacement of the exotherm into the temperature range of the denaturation endotherm occurs in response to changes that accelerate intermolecular aggregation and accompanying gelation.[45,46] The absence of a detectable exotherm in other studies using conventional calorimeters could be attributed to the much smaller sample mass, faster heat transfer, which may cause the exothermic heat flow from the slow aggregation process to be swamped by the endothermic heat flow from the more rapid denaturation process.[46] Then it is necessary to conduct additional calorimetric studies using wider temperature interval, lower heating rates, and different protein and salt concentration to differentiate the aggregation and denaturation process.

In conclusion, the addition of four continuous methiones in the variable region V of amaranth 11S globulins (*Ah*11Sr+4M), was enough to change the surface hydrophobicity−hydrophilicity observed by 3D structure analysis. These changes have as a result the decrease in protein solubility that affects its functional behavior. All amaranth 11S globulin versions developed gels at 90 °C, but *Ah*11Sr+4 M resulted in a middle strong gel. Hydrophobicity, charge distribution, disulfide/sulfhydryl content, and cavity size of proteins have been related to heat induced gelation.[47,48] The destabilization of tertiary structure by heat treatment could modify the surface behavior of proteins. High levels of hydrophobicity and electrostatic attractive forces and ability of disulfide bridges can promote gelation.

The present results established that protein engineering proved to be useful tools for designing novel amaranth 11S globulins with both nutraceutical and valuable functional properties that support their use in food products.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Summary of structure quality factors for unmodified *Ah*11Sr and modified *Ah*11Sr+4M. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: apbarba@ipicyt.edu.mx (A.P.B.R.); osuna.juan@hotmail.com (J.A.O.-C.).

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Notes

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