



Crystal structure of a major seed storage protein, 11S proglubulin, from *Amaranthus hypochondriacus*: Insight into its physico-chemical properties

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We dedicate this collaborative work to the memory of Prof. Utsumi who died on December 1, 2008

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ABSTRACT

Amaranth is a crop known for its high quality proteins. 11S Globulin is one of the most abundant and important storage proteins of the amaranth grain. Here, we report the crystal structure of amaranth 11S proglubulin at a final resolution of 2.28 Å. It belonged to the space group P6₃ with cell dimensions $a = b = 96.6$, $c = 75.0$ Å. It contains one asymmetric unit consisting of 372 residues and 100 water molecules. Disordered regions in the model approximately correspond to the variable regions of the 11S globulins. The structure has an extended α -helix and β -barrel domains at both N-terminal and C-terminal regions, which are characteristic of the 11S and 7S globulins. The three dimensional structure suggests that its high thermal stability is due to the cumulative effects of many factors and its good emulsifying property depended on the balance between its surface hydrophobicity and hydrophilicity.

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1. Introduction

Amaranth (*Amaranthus hypochondriacus* L.) is a dicotyledonous crop, which can supply grains and tasty leafy vegetables of high nutritional quality. It is one of the few non-grass species that is comparable to cereal crop as a source of dietary proteins. It is popular for its high amount of easily digestible protein, high level of lysine and sulphur-containing amino acids, good source of bioactive peptides (Silva-Sanchez et al., 2008; Vecchi & Anon, 2009), non-allergenic 11S globulin (Sinagawa-Garcia et al., 2004), and high resistance over adverse agronomical conditions like drought, heat and some pests (Huerta-Ocampo et al., 2011; National Research Council, 1984). For these reasons, amaranth has been receiving increased attention in research.

Globulin, albumin, and glutelin are the major protein fractions in amaranth seeds. Its globulin is composed primarily of the 11S globulin. 7S globulin is also present, but only in minor quantity.

Although 11S globulin and glutelin of amaranth differ in solubility, both have very similar subunit composition (Abugoch, Martinez, & Anon, 2003), exhibited cross-reactivity (Aphalo, Castellani, Martinez, & Anon, 2004), and have been suggested to be structurally homologous (Vasco-Méndez & Paredes-López, 1994). The presence of an intriguing albumin-2, which is peculiar to amaranth seeds, has also been documented. It can be extracted with water only after albumin-1 and globulins were extracted. Amaranth 11S globulin and albumin-2 have different solubility, but they have similar properties like molecular size and surface reactivity (Aphalo et al., 2004). Therefore, albumin-2 is probably an unprocessed 11S globulin or proglubulin (Barba de la Rosa, Herrera-Estrella, Utsumi, & Paredes-López, 1996; Molina, Circosta, Anon, & Petruccielli, 2008).

11S globulins are composed of three subunits in their pro-form (proglubulin). Co-translational signal peptide cleavage of the subunit prompts its molecular assembly in the endoplasmic reticulum that involves the association of three subunits in a head-to-tail-fashion. This proglubulin is transported into the protein storage vacuoles where they are cleaved by an Asn-Gly endopeptidase, resulting into acidic and basic chains. The cleavage favours the face-to-face association of two trimers resulting in mature hexamer globulins (Adachi, Takenaka, Gidamis, Mikami, & Utsumi,

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Table 1
Data collection and refinement statistics of proamaranth 11S globulin crystals.

Parameter	Data/statistics
<i>Diffraction data</i>	
X-ray source	Spring-8 (BL44XU)
Wavelength (Å)	0.90
Detector	MarMosaic 225 mm HE
Space group	P6 ₃
a, b, c (Å)	96.6, 96.6, 75.0
Resolution limit (Å)	50–2.28 (2.34–2.28)
Measured reflections	495,787 (40,532)
<I/σ(I)>	15.4 (2.2)
Unique reflections	88,303 (8624)
Completeness (%)	99.0 (99.3)
R _{sym} , R _{merge} (%) ^a	8.7 (46.7)
<i>Refinement</i>	
Resolution range (Å)	28.47–2.28 (2.42–2.28)
Used reflections	17,027 (2751)
Completeness (%)	98.1 (94.0)
Residues/waters	372/100
Ave. B-factor (Å ²)	28.43
Bond length r.m.s. (Å)	0.01
Bond angle r.m.s. (°)	1.22
R factor (%)	18.8 (24.7)
R _{free} (%)	24.9

^a $R_{sym}/R_{merge} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the observed intensity in the *i*th data set and $\langle I(hkl) \rangle$ is the mean intensity of reflection *hkl* over all measurements of *I(hkl)*.

2001; Adachi et al., 2003). The 11S proglobulin has a similar tertiary structure to that of 7S globulin, which forms a trimer. Both globulins contain an extended helix domain that links the subunits together and a β-barrel domain that serve as the core structure. The 7S globulins do not assemble into hexamers because they do not have the hydrophobic region that becomes exposed in 11S globulins after the cleavage between Asp–Gly (Tandang-Silvas et al., 2010a).

In this study, we report the crystal structure of amaranth 11S proglobulin expressed in *Escherichia coli* system. Correlations between the crystal structure and the amaranth globulin physico-chemical properties, such as its high thermal stability and good emulsifying ability are also discussed in this paper. Our findings can therefore aid in designing modifications for improved functionality of amaranth, which is also currently underway.

2. Materials and methods

2.1. Protein expression and purification

The cloned cDNA of amaranth 11S proglobulin (Barba de la Rosa et al., 1996) was expressed and purified using the method described in Tandang-Silvas et al. (2010a). Briefly, *E. coli* Rosetta-gami (DE3) containing amaranth 11S globulin cDNA in pET21d was cultured in Luria–Bertani (LB) broth containing 0.5 M NaCl for 20 h at 302 K after induction with 1 mM IPTG (isopropyl β-D-1-thiogalactopyranoside). Cells were then harvested, resuspended, and sonicated in buffer A [35 mM potassium phosphate, pH 7.6, 1.0 M NaCl, 1 mM EDTA, 0.1 mM (*p*-aminophenyl)-methanesulfonyl fluoride (*p*-APMSF), 1.2 μM leupeptin, 0.2 μM pepstatin A, 0.02% (w/v) NaN₃]. The resulting soluble fraction was subjected to a two-step purification involving an ammonium sulphate fractionation and size-exclusion chromatography using a Sephacryl S-300 26/60 column (GE Healthcare Life Sciences, NJ).

2.2. Crystallization and data collection

The hanging drop vapour diffusion technique was used to grow amaranth 11S proglobulin crystals at 293 K. The drop contained 5 μL of 10 mg/mL protein and 5 μL of the reservoir solution that consisted of 10% PEG3350 and 200 mM ammonium formate, pH 6.6. The harvested crystals were immersed for several minutes in

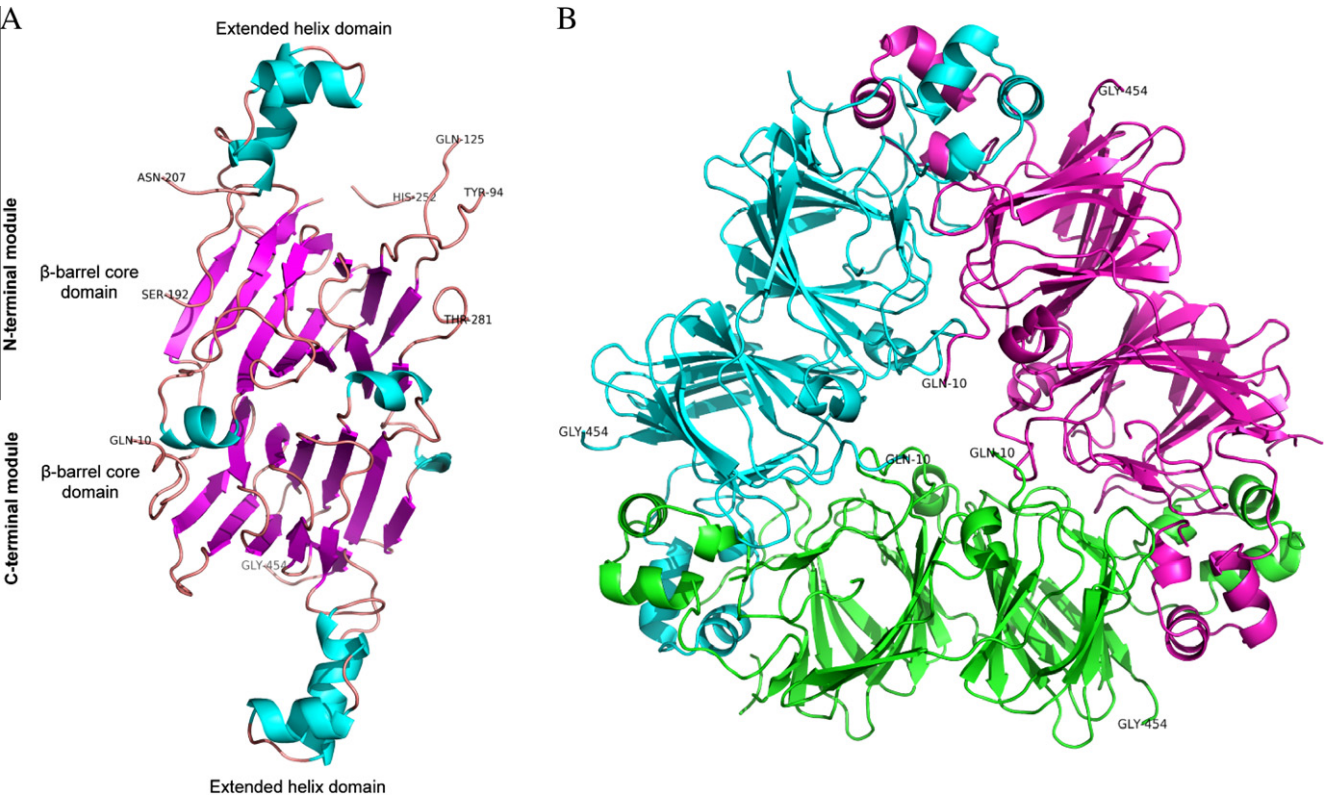
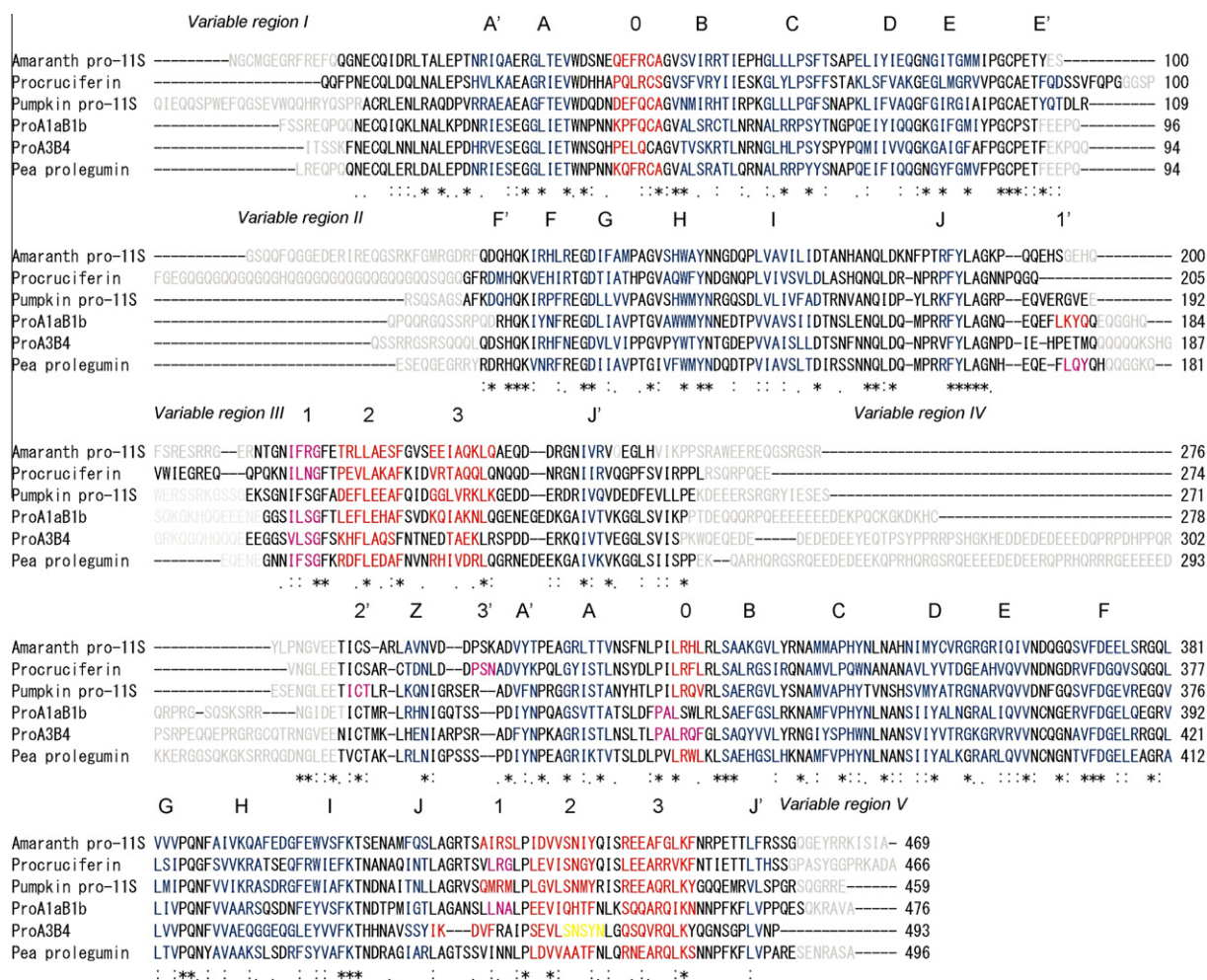


Fig. 1. Overall structure of the amaranth 11S proglobulin. (A) Monomer structure. (B) Trimer structure.



a cryoprotectant before flash-cooling in a stream of liquid nitrogen. The crystals that gave good diffraction in the in-house X-ray facility equipped with Bruker High Star detector were stored in liquid nitrogen and brought to SPring-8 (Japan). The diffraction images that gave up to 2.28 Å resolution were collected by CCD using beamline BL44XU, and processed using HKL2000 and Scalepack (Otwinowski & Minor, 1997).

The initial structure was determined by molecular replacement with one subunit of pumpkin 11S globulin structure (PDB entry 2E9Q) as a template using the program MOLREP (Brünger et al., 1998) followed by further refinement using REFMAC (Vagin et al., 2004). Both programs are part of the CCP4 package. Several rounds of manual refinement were done using Coot (Emsley & Cowtan, 2004), followed by Phenix refinement (Adams et al., 2010).

The structure of amaranth 11S proglobulin was deposited at the Protein Data Bank (PDB) and was assigned the PDB code 3QAC. The solved crystal structures that were used as reference in this study include that of procruciferin (PDB code 3KGL), pumpkin pro-11S

The refined model was validated with a Ramachandran plot calculated using PROCHECK (Vaguine, Richelle, & Wodak, 1999). Structural alignment was first carried out using the Superpose program (Krissinel & Henrick, 2004) under Coot. Secondary structures were determined using DSSP and validated using Coot. The surface accessibility of residues was calculated using both DSSP (Kabsch & Sander, 1983) and AREAIMOL (Lee & Richards, 1971). The cavity size was estimated by CASTp (Dundas et al., 2006). Accessible surface area was calculated using NACCESS (Hubbard & Thornton, 1993). The molecular surfaces were calculated and displayed using PyMOL. Electrostatic potentials were calculated and displayed using GRASP2 (Petrey & Honig, 2003).

Data collection and refinement statistics of amaranth 11S proglubulin crystal are summarized in [Table 1](#). The crystal structure

was determined at a final resolution of 2.28 Å. Its crystals belonged to the space group $P6_3$, with cell dimensions $a = b = 96.6$, $c = 75.0$ Å. A total of 284 (89.0%) of the amino acids were within the most favored region and 318 (99.6%) of the amino acids, excluding glycine (27), proline (17) and terminal residues (9), were within the allowed region of the Ramachandran plot of the refined model as calculated by PROCHECK, while 0.3% (1) of the amino acids were found in the outlier region. The outlier residue, Thr448, has a well-defined density map and is located in a sharp bend between the β -barrel and extended loop domain of the C-terminal module. The R and R_{free} values from the resulting model were 18.8% and 24.9%, respectively. The model contains one asymmetric unit consisting of 372 residues and 100 water molecules. Gly1–Gln9, Glu95–Phe124, Gly193–Arg206, Gln248, Val253–Glu280, Gln455–Ala465 are the residues in the highly disordered regions on the $2|F_o| - |F_c|$ map, and hence not included in the model. The five variable regions present among the 11S globulins approximately correspond to these disordered regions.

3.2. Amaranth 11S proglobulin structure

The ribbon representation of the solved structure (a monomer) is shown in Fig. 1A and is similar to the other 11S globulin structures. The monomer can be divided into two very similar modules, the N- and C-terminal modules, which can be separated by a pseudo-dyad axis (not shown). Each module contains the conserved β -barrel core (jelly roll-like) domain and an extended helix domain containing several helices. The β -barrel domain is formed by two β -sheets consisting of A'ABIDG and J'JCHEF segments (Fig. 2).

The amaranth proglobulin protomer is composed of three monomers, which are non-covalently linked in a head-to-tail manner (Fig. 1B) and has approximate dimensions of $90 \times 90 \times 40$ Å, which are comparable to the other 11S globulins ($89\text{--}95 \times 89\text{--}95 \times 39\text{--}41$ Å).

3.3. Structural comparison

The structure-based alignment of the amino acid sequences between amaranth 11S proglobulin and other 11S proglobulins is shown in Fig. 2. The α backbone superimposition of these proteins is shown in Fig. 3. Although there are minor differences, the amaranth pro-11S backbone structure is very similar to those of procruciferin, pumpkin pro-11S, proA1aB1b, proA3B4 and pea prolegumin. The amaranth pro-11S has root mean square deviations (RMSDs) of 1.28 Å compared with the 366 α atoms of procruciferin, 1.08 Å for the 364 α atoms of pumpkin pro-11S, 1.02 Å for the 354 α atoms of proA1aB1b, 1.11 Å for the 358 α atoms of proA3B4, and 1.02 Å for the 364 α atoms of pea prolegumin. Minor differences among 11S proglobulins (Figs. 2 and 3) are found at the N-termini, a region in between strands I and I', in strand 1, a region in between strands 3 and J' of the N-terminal modules, a region in between strands 2' and A' of the C-terminal modules and region after strand J towards the C-termini.

3.4. Structural factors and thermal stability

The mid-point denaturation temperature of amaranth 11S globulin varied with the type of buffer used as investigated by Chen and Paredes-Lopez (1997) who reported T_m values of 372 K, 372.8 K, and 376 K for water, 500 mM Tris–HCl (pH 8.0) containing 0.1 M NaCl, and 32.5 mM K_2HPO_4 –2.6 mM KH_2PO_4 (pH 7.5) containing 0.4 M NaCl, respectively. Although there is a small difference in the ionic strength used for T_m measurement, the T_m values obtained from amaranth 11S proglobulin are relatively high compared with the other 11S proglobulins (350.0–368.0 K) (Maruyama, Maruyama, Mikami, & Utsumi, 2004; Tandang-Silvas

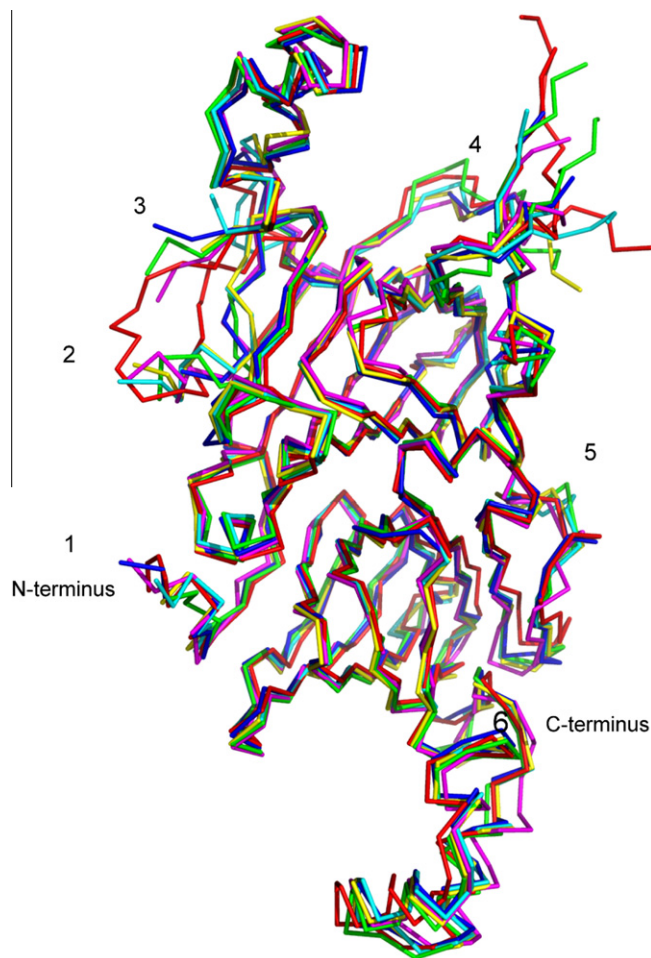


Fig. 3. α backbone superimposition of the amaranth 11S proglobulin with other 11S proglobulins. Blue: amaranth pro-11S; red: procruciferin; green: pumpkin pro-11S; yellow: proA1aB1b; magenta: proA3B4; cyan: prolegumin; 1: N-terminus; 2: region in between strands I and I'; 3: strand 1; 4: region in between strands 3 and J' of the N-terminal modules; 5: between strands 2' and A' of the C-terminal modules; 6: region after strand J towards the C-termini. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

et al., 2010b). The stabilizing effect of NaCl on 11S seed globulins has been corroborated by the other studies on rapeseed (Tandang, Adachi, Inui, Maruyama, & Utsumi, 2004), soybean, pea and faba bean (Kimura et al., 2008; Maruyama et al., 1999).

Some structural features cited to increase thermal stability of the 11S globulins are the high number of proline residues, reduced length of loops, and cavity size (Fukuda, Prak, Fujioka, Maruyama, & Utsumi, 2007; Maruyama et al., 2004). Proline residues in amaranth 11S proglobulin consist of 4.1% which is lower than soybean proA1aB1b, proA3B4, and procruciferin (6.1%, 7.3%, and 5.6%, respectively), but comparable to pumpkin 11S proglobulin and prolegumin (3.9% and 4.8%, respectively). In one subunit of amaranth 11S proglobulin, 59.6%, 30.3% and 10.1% of the residues assume a loop conformation, a β -strand conformation, and a helix conformation, respectively. The loop or disordered region in amaranth 11S proglobulin is comparable to the values (57–63%), reported for other pro-11S globulin crystal structures. The cavity size of amaranth 11S proglobulin is 4167.1 Å³, which is relatively small compared with other pro-11S globulins, whose value ranges from 4086.1 to 5620.3 Å³ (Tandang-Silvas et al., 2010b). High thermal stability of amaranth 11S proglobulin might be partly due to

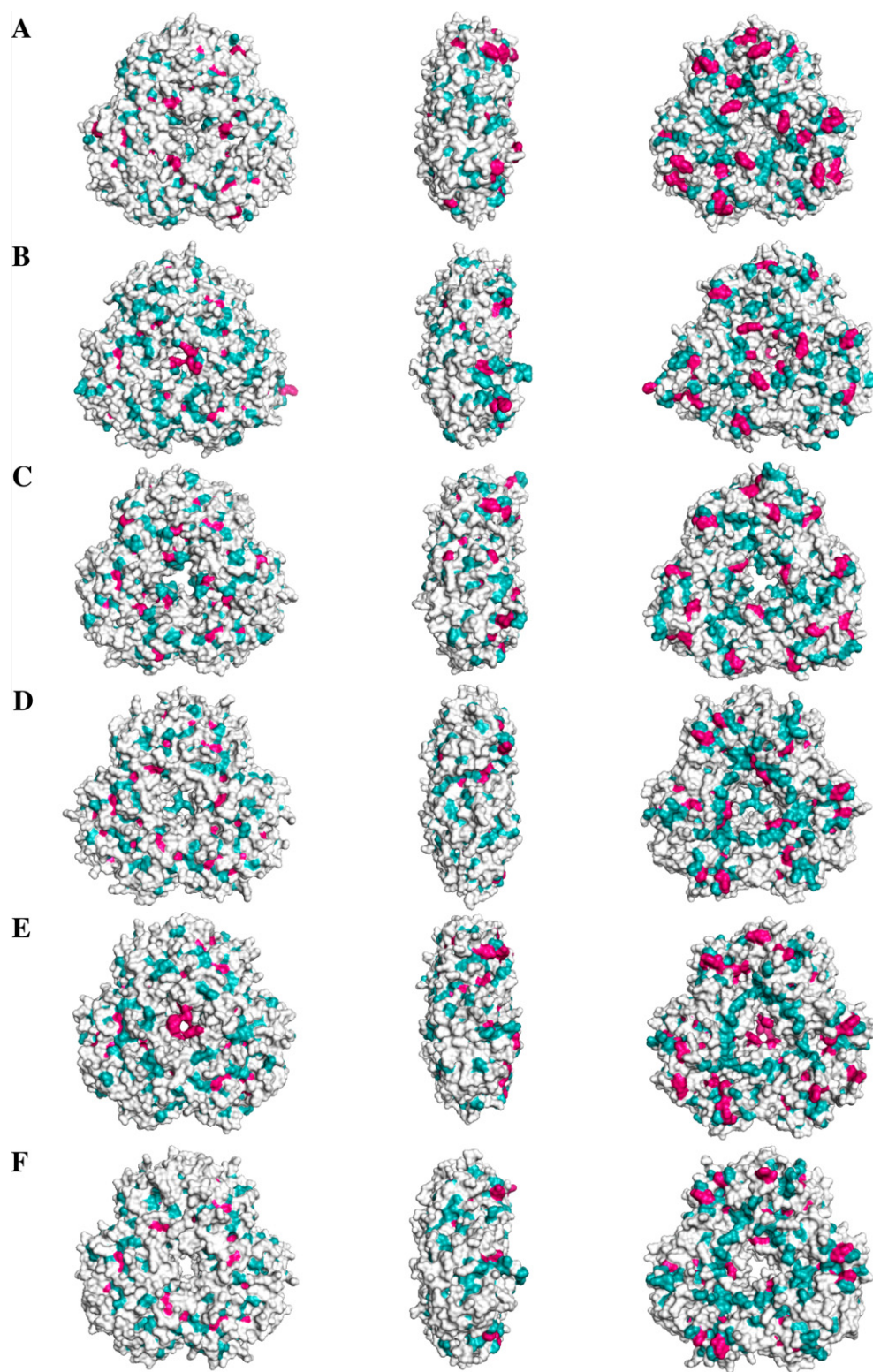


Fig. 4. Surface hydrophobicity of the 11S proglobulins. Distribution of aromatic (Phe, Tyr, and Trp) and aliphatic (Ala, Gly, Ile, Leu, Met, Pro, and Val) hydrophobic residues on the molecular surface are shown in pink and cyan, respectively. (A) Amaranth pro-11S; (B) procruciferin; (C) pumpkin pro-11S; (D) ProA1aB1b; (E) ProA3B4; (F) pea prolegumin. Structures are represented as white molecular surface models using Pymol. The centre and rightmost (IE face) diagrams represent views after 90° and 180° rotation around the vertical axis, respectively, of the leftmost diagram (IA face).

the smaller cavity size in its structure. We tried to find a correlation between thermal stability and the other structural features not mentioned above, such as, the number of intermonomer and/or intramonomer hydrogen bonds. However, no significant rela-

tionships could be established among the 11S proglobulins. This may be explained by the fact that thermal stability as well as protein stability in general, is brought about by the cumulative effects of all the above-mentioned structural features.

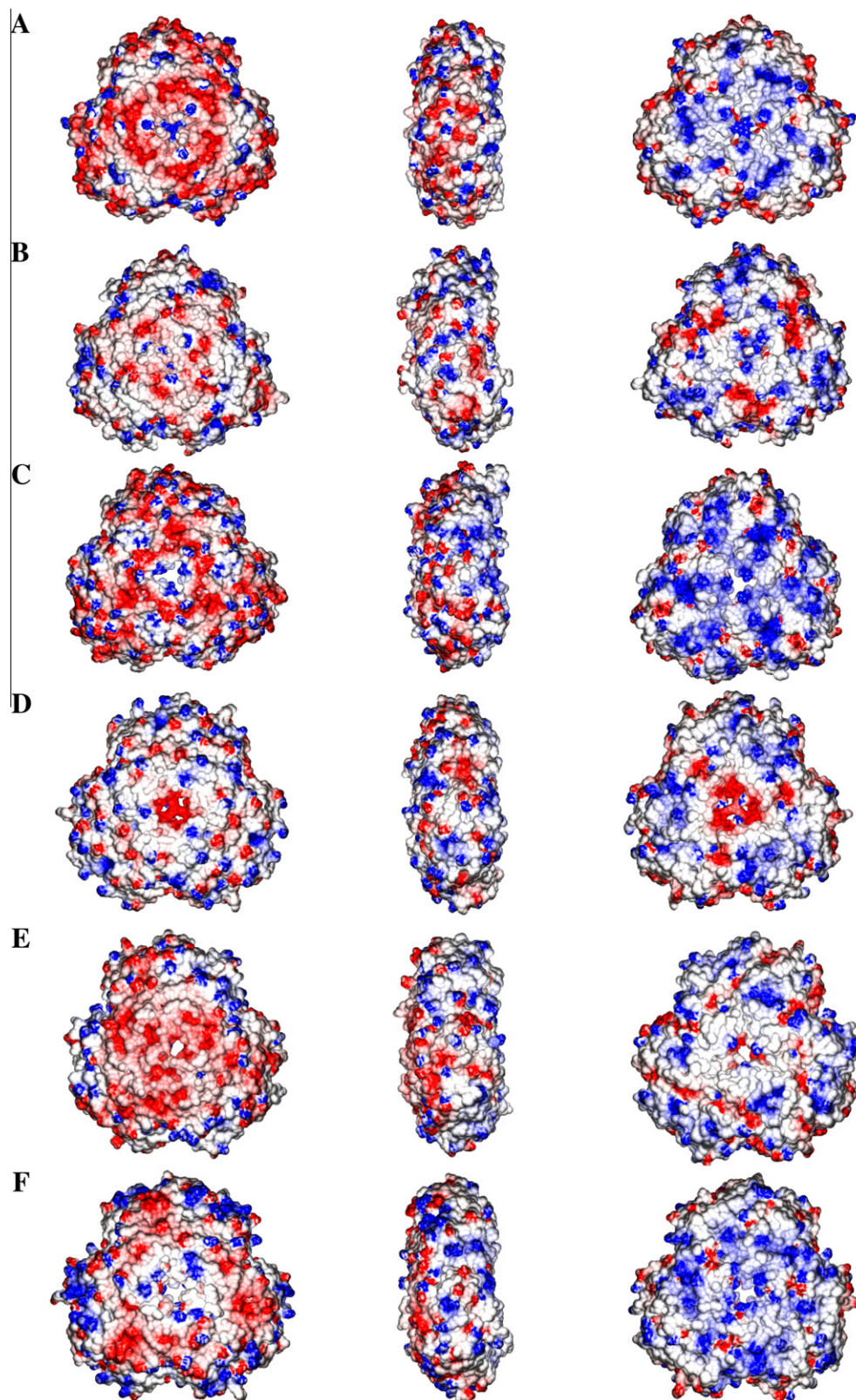


Fig. 5. Map of surface electrostatic potential of the 11S proglubulins. It was calculated using GRASP2 and rendered from the -10 (red; electronegative) to $+10$ kT (blue, electropositive) range, where k is the Boltzmann constant and T is the absolute temperature (K). (A) amaranth pro-11S; (B): procruciferin; (C) pumpkin pro-11S; (D) ProA1aB1b; (E): ProA3B4; (F): pea prolegumin. The views were rendered in the same manner as those of Fig. 4. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

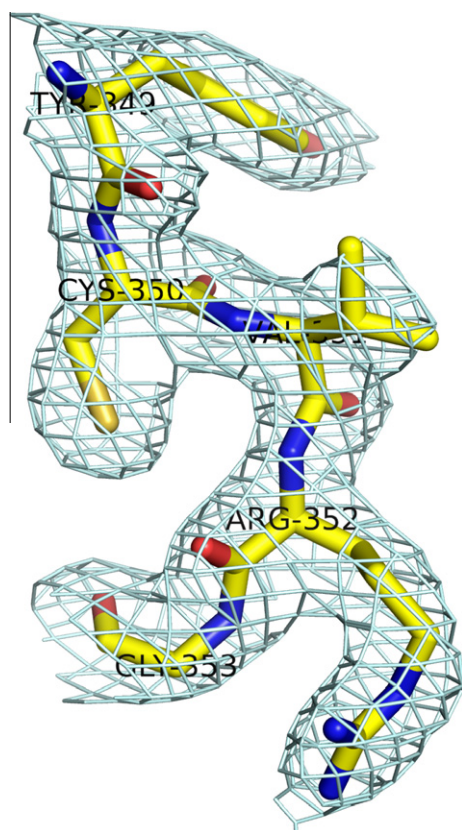


Fig. 6. Tyr349–Gly353 and its $2F_o - F_c$ map contoured at 1.0σ . Atoms are shown in different colours: C = yellow; O = red; N = blue; and S = orange. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.5. Surface character, emulsifying property, and aggregation tendency

Amaranth 11S proglobulin has been reported to possess good emulsifying ability (Barba de la Rosa et al., 1996; Konishi & Yoshimoto, 1989). Many studies have revealed that a protein's emulsifying ability is actually dependent on the balance between the surface hydrophobicity and hydrophilicity (solubility or surface charge) (Prak, Nakatani, Maruyama, & Utsumi, 2007; Tang & Sun, 2010; Voutsinas, Cheung, & Nakai, 1983). The 11S proglobulins used in this study generally have similar surface hydrophobicity, as shown in Fig. 4. The solved structure of amaranth 11S proglobulin revealed that it has the lowest distribution of hydrophobic residues on its surface (22.5%) relative to the other proglobulins (23.1–28.2%). Amaranth 11S proglobulin also possesses a highly concentrated distribution of positive and negative surface charge on its two sides that is generally comparable with the other 11S proglobulins, which accounts for its hydrophilicity (Fig. 5). This balance of hydrophobic and hydrophilic surfaces in amaranth 11S protein might account for its good emulsifying property.

Amaranth 11S proglobulin also has a tendency to aggregate at low temperature and at low ionic strength. It has been observed to be soluble only at a buffer pH near neutral (i.e., pH 7.6) and at medium to high ionic strength (i.e., 0.5–1.0 M NaCl) (Tandang-Silvas et al., 2010a). Repulsive and attractive electrostatic interactions are some of the factors that drive protein aggregation (Majhi et al., 2006; Piazza, Iacopini, & Galliano, 2002). The presence of a highly concentrated distribution of surface charge in this protein could explain this phenomenon and only by increasing the buffer ionic strength can the aggregation be prevented.

Amaranth 11S proglobulin has five cysteine residues at position numbers 14, 47, 90, 283 and 350. A disulphide bond in an acidic chain is formed between Cys14–Cys47 with an absolute ASA of 11.58 and 13.96 Å². On the other hand, a disulphide bond between acidic and basic chains is formed between Cys90–Cys283 with an absolute ASA of 17.15 and 40.46 Å². It seems logical that Cys283 has relatively higher ASA because it is close to the processing site for the cleavage between acidic and basic chains. Cys350, which is supposed to give a free sulfhydryl group, has an ASA of 0 Å², and is involved in the β -strand formation (Fig. 6). Other 11S globulins have either Ala or Val at this position. In the previous report (Tandang-Silvas et al., 2010a), low temperature and low ionic strength buffer lead to the aggregation of amaranth 11S proglobulin. The stable disulphide bonds described above suggest that the aggregation of amaranth 11S proglobulin at low ionic strength and low temperature would not be likely due to unfavorable disulphide bond formation between the trimer molecules.

4. Conclusion

The refined structure of amaranth 11S proglobulin consisted of a monomer in one asymmetric unit with 372 residues. Each monomer consisted of two jelly roll-like β -barrel domains and two extended helix domains. There were five missing or disordered regions similar to the other 11S globulins. The three dimensional structure suggests that (1) its high thermal stability is brought by cumulative effects of many factors, (2) its surface hydrophobicity resembles those of the other 11S proglobulins, and (3) its high emulsifying ability might be due to the interplay of its surface hydrophobicity and hydrophilicity or relative solubility.

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