Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

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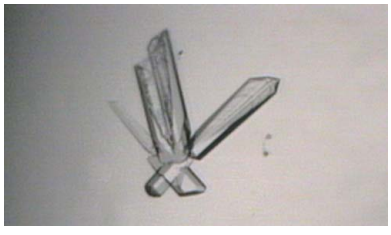
Received 19 May 2010
Accepted 2 June 2010

This work is dedicated to the memory of Professor Utsumi who died on 1 December 2008. He was instrumental in making this collaborative work possible.



Expression, purification and preliminary crystallization of amaranth 11S proglobulin seed storage protein from *Amaranthus hypochondriacus* L.

11S globulin is one of the major seed storage proteins in amaranth. Recombinant protein was produced as up to ∼80% of the total bacterial protein using *Escherichia coli* Rosetta-gami (DE3) containing pET21d with amaranth 11S globulin cDNA. The best expression condition was at 302 K for 20 h using LB medium containing 0.5 *M* NaCl. The recombinant protein was easily separated from most of the *Escherichia coli* proteins by precipitation with 0–40% ammonium sulfate solution. It formed aggregates at low temperature and at low salt concentrations. This behaviour may imply that it has a more hydrophobic nature than other 11S seed globulins. The crystals diffracted to 6 Å resolution and belonged to space group $P6_3$, with unit-cell parameters $a = b = 97.6$, $c = 74.8$ Å, $\gamma = 120.0^\circ$. One subunit of a trimer was estimated to be present in the asymmetric unit, assuming a V_{sol} of 41%. To obtain the complete structure solution, experiments to improve crystallization and flash-cooling conditions are in progress.

1. Introduction

11S globulin, albumins and glutelins are the major seed storage proteins of amaranth (*Amaranthus hypochondriacus* L.; Konishi *et al.*, 1985; Barba de la Rosa *et al.*, 1992). 7S globulin is present in minor quantities (Molina *et al.*, 2008).

The pro-form of legumins or 11S globulins is composed of three subunits, while the mature form consists of two trimeric molecules. The N-terminal domain (30 kDa) and the C-terminal domain (20 kDa) of a subunit are linked by a conserved inter-chain disulfide bridge. This linkage and the five variable regions in the 11S globulins are not important in maintaining their tertiary structure (Utsumi *et al.*, 1993; Adachi *et al.*, 2001, 2003). Pro-11S globulins are synthesized in the rough endoplasmic reticulum, where the co-translational signal peptide cleavage and subsequent trimer assembly occur. The cleavage of the two domains by a vacuolar processing enzyme in the protein-storage vacuole triggers the formation of mature globulins (Dickinson *et al.*, 1989). This enzyme is absent in *Escherichia coli*, so that only the pro-form can be obtained.

Proamaranth 11S globulin has previously been expressed as inclusion bodies and attempts to express it in a soluble form resulted in minimal yields (Osuna-Castro *et al.*, 2000; Molina *et al.*, 2008). The most efficient expression yield was 0.076 g per litre using Terrific broth (TB) and 75% of this was soluble (Medina-Godoy *et al.*, 2004). Here, we report the accumulation of a higher amount of soluble protein using *E. coli*, a two-step purification process and preliminary crystallization experiments. To obtain its complete structure, further studies to identify better crystallization and flash-cooling conditions are in progress. We hope to provide the molecular basis of the reported properties of amaranth 11S proglobulin such as its thermal stability and emulsifying abilities (Konishi & Yoshimoto, 1989).

2. Materials and methods

2.1. Protein expression and purification

Expression using *E. coli* Rosetta-gami (DE3) with amaranth 11S globulin cDNA in pET21d (Barba de la Rosa *et al.*, 1996) was con-

ducted using the following culture media: (i) TB containing 1 *M* sorbitol, (ii) Luria broth (LB) containing 0.17 *M* NaCl and (iii) LB containing 0.5 *M* NaCl. Expression was allowed to proceed for 20 or 40 h at either 293 or 302 K after induction with IPTG. For large-scale protein expression, the shaker was set to 90 rev min^{-1}. The total, soluble and insoluble proteins obtained after cell sonication in buffer *D* (35 m*M* potassium phosphate pH 7.6, 1 *M* NaCl, 1 m*M* EDTA, 10 m*M* β-mercaptoethanol, 0.1 m*M* p-APMSF, pepstatin and leupeptin) at 277 K were applied onto SDS–PAGE gel. The soluble proteins were precipitated in 0–40% saturated ammonium sulfate solution at 277 K. The redissolved precipitate was applied onto a Sephacryl S-300 26/60 column and eluted with buffer *D* at a flow rate of 1 ml min^{-1} at room temperature. The fraction that eluted at 170–180 min was collected, concentrated and used for crystallization.

2.2. Crystallization and preliminary data collection

Several crystal screening kits from Sigma, Hampton Research and Emerald BioSystems were initially used with the sitting-drop vapour-diffusion method in a 96-well Intelli-plates. Several precipitants such as 2-propanol, PEG 400, PEG 1000, PEG 3000, PEG 3350 and 2-methyl-2,4-pentanediol were identified as promising. The hanging-drop vapour-diffusion technique was used to grow larger crystals at 293 K. Different precipitants and protein concentrations were tested. The drop contained 5 µl 4–15 mg ml^{-1} protein solution and 5 µl reservoir solution. Several of the crystals that were grown were immersed in solutions containing cryoprotectants such as glycerol and 2-methyl-2,4-pentanediol before flash-cooling and checking their diffraction images using an in-house Bruker HI-STAR detector. The screened crystals were stored in liquid N_2 and transported to SPring-8, Japan. Crystal diffraction data were collected in a nitrogen-gas stream at 100 K using an ADSC Quantum 210 CCD detector adjusted to a crystal-to-detector distance of 330.0 mm and a wavelength of 1.00 Å on the BL38B1 beamline. For each frame, the oscillation range was 1° and the exposure time was 20 s. A total of 247 frames were collected and the images were processed using *HKL*-2000 (Otwinowski & Minor, 1997) and *SCALEPACK*. Molecular replacement was performed with *MOLREP* (Vagin & Teplyakov, 1997) from the *CCP*4 package (Collaborative Computational Project, Number 4, 1994) using a subunit of pumpkin 11S globulin (PDB code 2e9q;

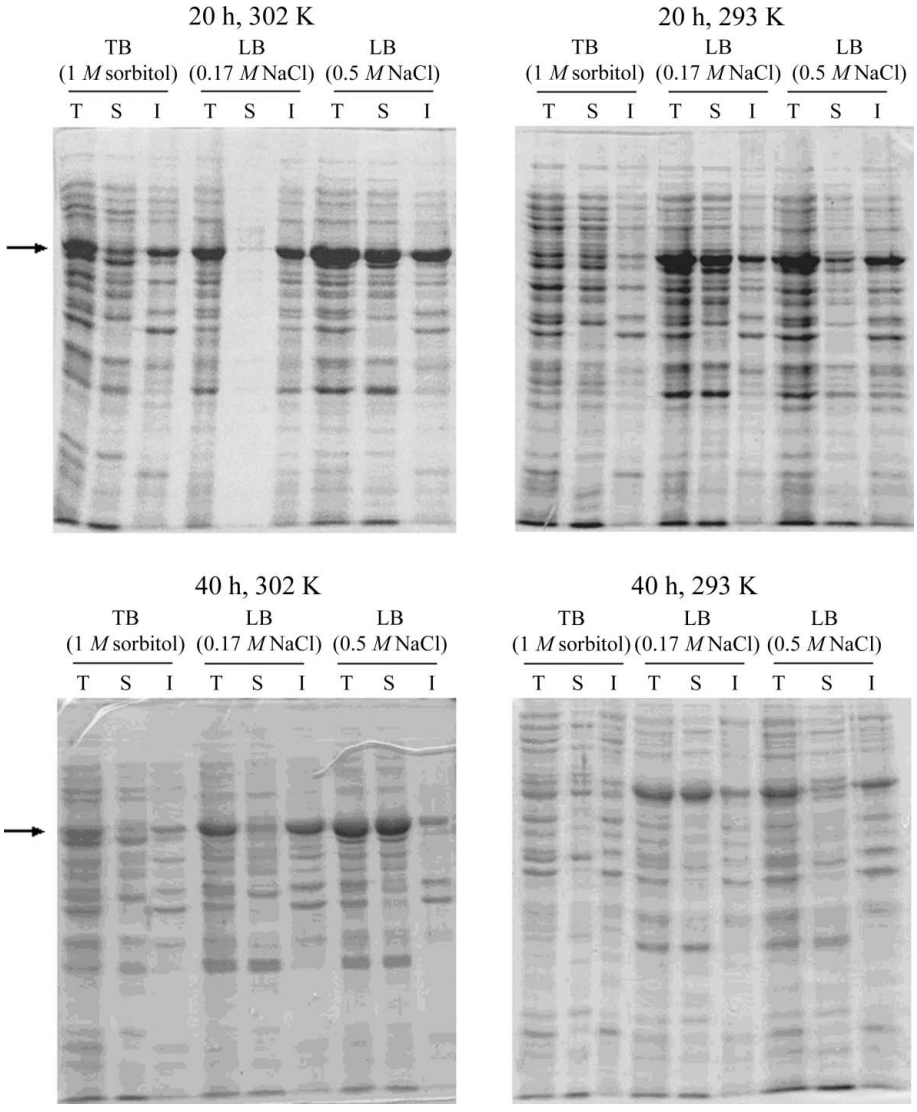


Figure 1
Screening for conditions suitable for the expression of amaranth 11S proglobulin (indicated by an arrow). T, S and I refer to total, soluble and insoluble fractions, respectively, for each 5 µg protein sample.

Tandang-Silvas *et al.*, 2010) as a model. The model was further refined using the rigid-body mode of *REFMAC*5 (Vagin *et al.*, 2004), also from the *CCP*4 package, followed by refinement in *PHENIX* (Adams *et al.*, 2002).

2.3. N-terminal amino-acid sequencing

The N-terminal amino-acid sequence of the purified protein was verified using a Procise 492 Protein Sequencer (Applied Biosystems).

2.4. Protein measurement

The Protein Rapid Assay Kit from Wako was used to measure the protein concentration in solution. Bovine serum albumin was used as standard.

3. Results and discussion

3.1. Expression and purification

The best expression condition was at 302 K for 20 h in LB containing 0.5 *M* NaCl (Fig. 1). The desired protein comprised about 80% of the total *E. coli* proteins and about 60% of this amount was soluble. The yield of the pure protein, as verified by N-terminal sequencing, was 0.06–0.08 g per litre of culture after the two-step purification process. Neither an increase in the expression time to 40 h nor the use of TB containing 1 *M* sorbitol (Fig. 1) improved the expression level. This was despite previous reports that richer media (Moore *et al.*, 1993) and sorbitol (Blackwell & Horgan, 1991) could increase the expression yield. Increasing the NaCl concentration in LB from 0.17 to 0.5 *M* significantly improved the protein solubility at 302 K. This result is in agreement with previous reports on the enhancing effect of NaCl on protein expression (Chopra *et al.*, 1994; Tandang *et al.*, 2004). However, a similar improvement was not observed at 293 K (Fig. 1), indicating that a lower expression temperature does not always favour a high yield of soluble protein.

Fig. 2 shows that most of the protein precipitated in 0–40% ammonium sulfate solution. Procruciferin and pumpkin pro-11S globulin, which share 44% sequence homology to amaranth 11S proglobulin, also precipitated in this ammonium sulfate range (Tandang-Silvas *et al.*, 2010). Soybean proglycinin A1aB1b and pea prolegumin, which share 41% and 39% sequence homology to amaranth 11S proglobulin, remained soluble in 40% ammonium sulfate solution. The unconserved amino acids are most likely to be exposed at the surface of the molecule, affecting the surface hydrophobicity, as manifested by their solubility in ammonium sulfate solution.

Overnight incubation of the ammonium sulfate precipitate fraction resuspended in buffer containing 0.15 *M* NaCl at 277 K resulted in about 90% precipitation (Fig. 3). Increasing the NaCl level to 0.4 *M* decreased the amount of precipitation to only about 20%. Therefore, anion-exchange chromatography was skipped in this study owing to aggregation at low salt concentrations. Gel filtration using buffer containing 1.0 *M* NaCl was instead performed to remove ammonium sulfate and to further purify the sample before proceeding with the crystallization experiments. Higher ionic strength increases protein solubility either by preventing charge–charge associations or by creating an environment that will not favour hydrophobic interactions. Degradation was also inhibited by high ionic strength buffer (Fig. 3). Keeping the protein at temperatures lower than room temperature (209 K) resulted in aggregation even when the buffer

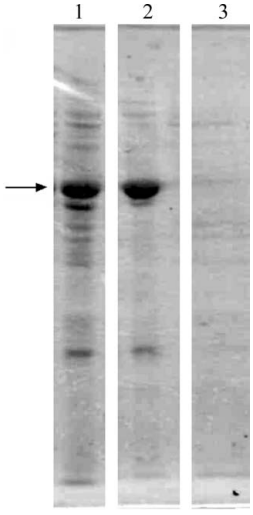


Figure 2
Purification of amaranth 11S proglobulin. Lane 1, total soluble proteins; lane 2, 0–40% ammonium sulfate precipitate; lane 3, 0–40% ammonium sulfate supernatant.

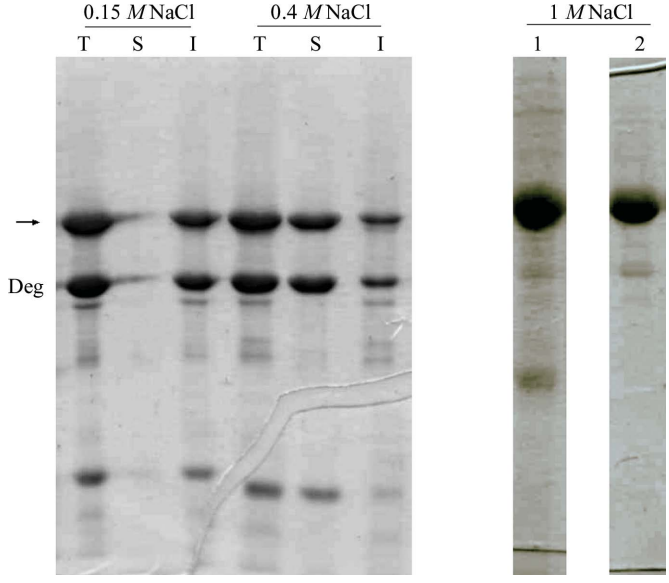


Figure 3
Effect of NaCl on the aggregation behaviour of amaranth 11S proglobulin. T, S and I refer to total, soluble and insoluble fractions, respectively. The arrow and Deg refer to intact and degraded proteins, respectively. Lane 1, before gel filtration; lane 2, after gel filtration.

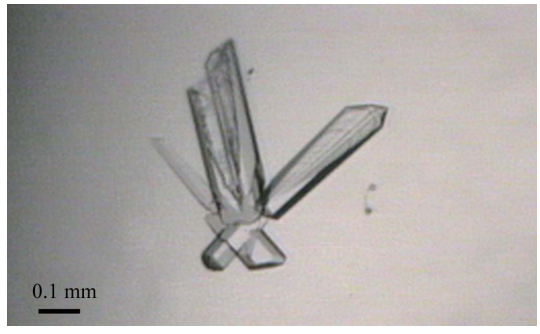


Figure 4
Amaranth 11S proglobulin crystals grown using 10 mg ml^{-1} protein solution in 10% PEG 3350 and 0.2 *M* ammonium formate pH 6.6 at 293 K.

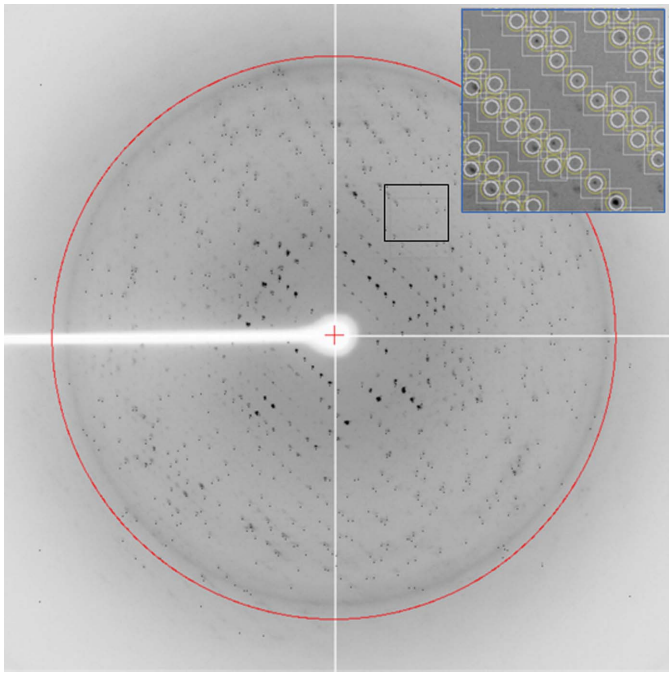


Figure 5
Diffraction image of a amaranth 11S proglobulin crystal. The outer red circle corresponds to 6.0 Å resolution. The inset shows a magnified fragment with simulated spots from the unit-cell parameters.

contained 1 *M* NaCl (data not shown). Similar behaviour has been reported by Konishi *et al.* (1985).

3.2. Preliminary crystal data collection

Small crystals appeared and grew to dimensions of about 0.5 × 0.2 × 0.1 mm within a few days of setup (Fig. 4). They were very fragile and usually cracked when flash-cooled. Also, ice rings usually appeared even when several cryoprotectants were used. A representative crystal diffraction image is shown in Fig. 5. The crystals belonged to space group $P6_3$, with unit-cell parameters $a = b = 97.621$, $c = 74.859$ Å, $\gamma = 120.0^\circ$. One subunit of a trimer was estimated to be present in the asymmetric unit, assuming a V_{sol} of 41%. In 247 frames, a total of 10 572 reflections corresponding to 1050 unique reflections were collected with 99.2% completeness and an R_{merge} of 0.056 to 6.0 Å resolution. The present model contains one subunit consisting of 453 amino-acid residues with $R = 0.22$ and $R_{free} = 0.41$ for data in the resolution range 30–6 Å. We are searching for improved crystallization and flash-cooling conditions in order to obtain the complete structure solution.

This work was supported by Mexico–Japan fund Ref. J000.363 S-2100 awarded to APBDLR and NM. X-ray data collection on BL38B1 of SPring-8 was carried out under JASRI proposal No. 2009B1399.

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