Overexpression, Purification, and in Vitro Refolding of the 11S Globulin from Amaranth Seed in *Escherichia coli*

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An amarantin 11S globulin cDNA encoding one of the most important storage proteins of amaranth seeds, with a high content of essential amino acids, was expressed in *Escherichia coli*. A good level of expression of recombinant amarantin with a molecular weight of 59 kDa was obtained. The recombinant protein was extracted by ammonium sulfate precipitation and purified to homogeneity using ion-exchange chromatography and reversed phase high-performance liquid chromatography. The expressed protein exhibited electrophoretic, immunochemical, and surface hydrophobicity properties similar to those of native amarantin from amaranth seed. Also, the recombinant protein was refolded in vitro using two different methods.

Keywords: *Storage protein; amarantin; heterologous expression; recombinant amarantin; expression induction; immunodetection; in vitro refolding*

INTRODUCTION

Seeds are the most important source of dietary proteins for humankind and livestock; their high content of storage proteins provides a supply of amino acids for use during germination and seedling growth. In fact, the storage proteins are major determinants of the nutritional and food-processing qualities of the major seed crops (Shewry, 1995, 1998). However, the prolamin storage proteins of cereals are deficient in lysine and threonine or tryptophan, whereas the saline-soluble globulins of legumes are deficient in the sulfur-containing amino acids methionine and cysteine (Shewry, 1995).

Amaranth is a pseudocereal producing high yields of nutritious and edible grainlike seeds. It also has nutraceutical characteristics as initially recognized by Mayans and Aztecs, with the result it has been termed by some authors "the food crop of the 21st century" (Lehmann, 1996; Chaturvedi et al., 1997; Guzmán-Maldonado and Paredes-López, 1998). With a protein level of ∼13−19%, the amaranth crop compares well with commercial cereals (∼10% protein) and contains more lysine and sulfur-containing amino acids than cereals and some important legumes. In fact, its essential amino acid balance is, in general, better than that of both types of seeds (Barba de la Rosa et al., 1992a; Segura-Nieto et al., 1994; Romero-Zepeda and Paredes-López, 1996).

Globulins are a major amaranth protein fraction, with the 11S globulin, called amarantin, representing 90 and 18.6% of the total globulins and total seed proteins, respectively (Romero-Zepeda and Paredes-López, 1996; Marcone, 1999). Consequently, excellent nutritional properties have been largely ascribed to this specific protein (Barba de la Rosa et al., 1992a,b; Segura-Nieto et al., 1994, 1999; Romero-Zepeda and Paredes-López, 1996; Marcone, 1999). In addition, amarantin also possesses remarkable heat stability and emulsifying properties and is therefore expected to be useful as a potential emulsifier with high nutritional value (Konishi and Yoshimoto, 1989). However, it shows lower solubility in an acidic pH (4.5−6.8) due to the effect of its isoelectric point, which lies within this pH range (Konishi and Yoshimoto, 1989; Marcone and Yada, 1992).

Work from our laboratory (Barba de la Rosa et al., 1992a,b; Segura-Nieto et al., 1994, 1999; Romero-Zepeda and Paredes-López, 1996; Chen and Paredes-López, 1997) and other studies (Konishi et al., 1985; Marcone and Yada, 1991) have shown that amarantin is a homohexameric molecule with an apparent molecular weight ∼300−400 kDa comprising subunits of 59 kDa, each of which consists of an acidic polypeptide (34−36 kDa) and a basic polypeptide (22−24 kDa) linked by a disulfide bridge. Recently, Barba de la Rosa et al. (1996) isolated and cloned a cDNA encoding the amaranth 11S globulin. Analyses of the deduced protein primary structure support the hypothesis that the protein is synthesized as a single polypeptide precursor (named preproamarantin) consisting of acidic and basic polypeptides together with a hydrophobic signal peptide (Shewry, 1995).

Moreover, although amarantin appears to have features similar to those of other 11S-type globulins, it has the advantage of containing a higher level of essential amino acids, which is closer to optimum required amounts for humans established by international health organizations (FAO/WHO, 1991; Barba de la Rosa et al., 1996).

A number of strategies are being considered to ad-

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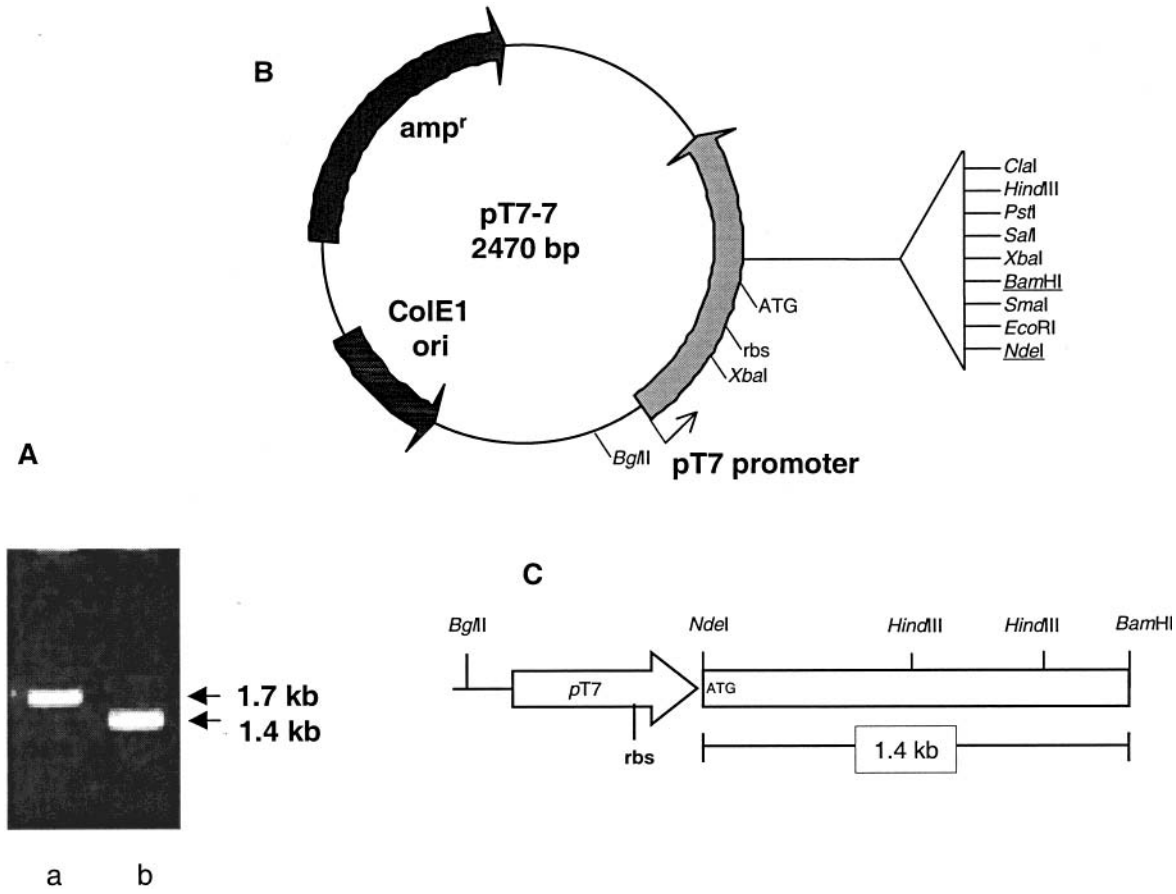

Figure 1. Amarantin cDNA fragments used to construct pSPORT1-AMAR and pT7-AMAR, with and without signal peptide, respectively: (A) track a, 1.7 kb *Not*I/*Sal*I amarantin cDNA purified from pSPORT1-AMAR with signal peptide, and track b, 1.4 kb PCR amplified amarantin fragment signal peptide removed by PCR; (B) plasmid map of pT7-7, which contains a T7 polymerase promoter, the gene enconding resistance to the antibiotic ampicillin, the ColE1 origin of replication, a strong ribosome-binding site (rbs) and start codon (ATG) upstream of the polylinker sequence; (C) restriction map obtained with particular restriction enzymes from 1.4 kb amarantin fragment and promoter genes from pT7-AMAR.

dress the problem of seed processing and nutritional quality. One approach is to modify the existing proteins of seeds in order to improve their balance of essential amino acids and functional properties simultaneously. Alternatively, it may be possible to transfer genes encoding proteins with high nutritional−functional value to crops of traditional agronomic importance (Sindhu et al., 1997; Shewry, 1998; Rooke et al., 1999). Currently, biotechnology and molecular biology offer an opportunity to effect improvements through rational modification−expression of genes or cDNAs coding for heterologous proteins with high nutritional−functional quality, using protein-engineering techniques (Shewry, 1998).

Protein engineering should not only facilitate the modification of protein currently used for food but also allow the analysis of other novel proteins, which should optimize their utilization in either traditional or new foods (Sindhu et al., 1997; Shewry, 1998). Amarantin is an excellent candidate to be overexpressed in transgenic plants of important crops such as maize or rice to improve their properties as protein ingredients or their nutritional value.

To achieve these objectives, it is necessary to establish a high-level heterologous expression system for native and bioengineered amarantins, to allow the rapid production of large quantities of homogeneous protein. This would also allow us to determine whether the recombinant globulins are able to acquire the proper molecular conformation and exhibit the expected food functionalities. For these reasons, we report the expression of amarantin cDNA in *Escherichia coli* and also its immunodetection, purification, and in vitro refolding.

MATERIALS AND METHODS

Plant Material. Mature seeds of *Amaranthus hypochondriacus* (Mercado type) were kindly provided by the Instituto Nacional de Investigaciones Forestales y Agropecuarias (INIFAP), Chapingo, Mexico. They were ground in a mill (Tekmar) with a mesh screen, and the whole flour was defatted by extraction with hexane (1:10 w/v) by continuous stirring during 24 h at 4 °C. The defatted meal was air-dried at room temperature and stored at 4 °C until use (Chen and Paredes-López, 1997).

Construction of Amarantin Expression Plasmid, pT7-AMAR. The full-length amarantin cDNA was released from pSPORT1-AMAR (Barba de la Rosa et al., 1996) by restriction digestion with *Not*I and *Sal*I (Figure 1). PCR was used to amplify a portion of the cDNA amarantin lacking the endogenous (N-terminal) signal sequence. This was carried out using the gel-purified *Not*I/*Sal*I fragment as a template, with two gene-specific primers. These primers, AMX.for (5′ tg cat **atg** ggg tgt atg ggt gaa gg 3′) and AMX.rev (5′ at gga tcc **tta** ggc aat gct gat tt 3′), annealed either to the start of the mature form of amarantin (AMAR.for) or to the stop codon (AMAR.rev), as indicated in bold. In the case of AMX.for, an initiating methionine was also introduced into the sequence (via the *Nde*I site). Appropriate restriction sites facilitating the cloning of the amplified PCR product into the pT7 RNA-polymerase-dependent expression vector were also incorpo-

rated into the 5′ (noncomplementary) portion of the primers and are underscored (AMX.for, *Nde*I; AMX.rev, *Bam*HI). Thus, the 1.4 kb amarantin PCR fragment was therefore cloned into the *Nde*I/*Bam*HI-cut pT7-7 expression vector **(**Novagen) to generate a recombinant amarantin expression plasmid designated pT7-AMAR (Figure 1).

The pT7 series of vectors allow for the expression of high levels of a protein from a target cDNA sequence under the control of Φ 10, a T7 RNA polymerase-specific promoter. *E. coli* strain BL21 (DE-3) **(**$\mathbf{F^- \ ompT \ r_B^- \ m_B^-}$**,** Novagen**)** carries the gene for T7 RNA polymerase in the chromosome, which is in turn controlled by the *lac*UV5 promoter. Expression of the T7 RNA polymerase and hence transcription of the target sequence can be induced by isopropyl *β*-D-thiogalactopyranoside (IPTG) (Studier, 1990).

Expression and Detection of Amarantin in *E. coli*. The resultant construct, which also contains an ampicillin resistance gene, was transformed into the expression *E. coli* strain BL21 (DE-3). To optimize the expression conditions, the bacterial cells were grown on LB medium (1% Bacto tryptone, 0.5% Bacto yeast extract, and 110 mM NaCl, pH 7.0) containing carbenicillin (100 *μ*g/mL) at either 30 or 37 °C until an OD_{600} between 0.4 and 0.6 was reached. At this point, IPTG was added to a final concentration of 0.4 mM. Cells were incubated for 24 h in a shaking bath at 200 rpm with controlled temperature. Samples of cells taken at different times (0, 3, 6, 9, and 12 h) after induction were harvested by centrifugation. Total cell pellets were resuspended in SDS sample buffer [62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 0.002% (w/v) bromophenol blue, and 5% (v/v) 2-mercaptoethanol] and analyzed by SDS-PAGE according to the Laemmli (1970) system with 12% acrylamide gels, which were stained with Coomassie Brilliant Blue G250. To estimate the molecular weight of the expressed protein, a kit of molecular weight standards was used (Wide Range Color Markers, Sigma).

Antibody Preparation and Immunoblot Analysis. Rabbit polyclonal antibodies were raised against the whole amarantin subunit purified from amaranth seeds. Immunochemical detection of the expressed protein was by western blot analysis according to the method of Fido et al. (1995). Briefly, after electrophoresis, gels were incubated in transfer buffer (25 mM Tris-HCl, pH 8.3, 192 mM glycine, and 20% methanol) and then were transferred to a PVDF membrane using a wet electroblotting apparatus at 50 V for 30 min (Mini Trans-Blot R electrophoretic transfer cell, Bio-Rad). The membrane was incubated in blocking solution [0.1% (w/v) bovine serum albumin (BSA) and 5% Marvel skim milk in Tris-buffered saline (TBS, 20 mM Tris-HCl, pH 7.5, 0.5 M NaCl)] and then washed twice with 0.05% Tween 20 in Tris-buffered saline (TTBS). The membrane was then shaken overnight in a solution containing 1% BSA in TTBS and a 1:250 dilution of the anti-amarantin antibody. After two washing steps in TTBS, the anti-rabbit antibody conjugated to alkaline phosphatase was added. Finally, the enzyme−substrate reaction was performed by incubating the membrane in carbonate buffer (0.1 M $NaHCO_3$, pH 9.8, 1.0 mM $MgCl_2$) containing *p*-nitroaziltetrazole chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate toluidine salt (BCIP). The color was developed after a few minutes, and the reaction was stopped by with washing in distilled water.

Purification of Expressed Protein. Expressed amarantin was isolated and purified from pT7-AMAR/BL21 (DE-3) *E. coli* cells using the expression conditions as described above. Cells were grown on LB broth (1 L in 2-L flasks) treated with carbenicillin (100 *μ*g/mL) and induced with 0.4 mM IPTG. After incubation for 6 h at 30 °C and faster shaking at 250 rpm, a total of 10 L of culture was centrifuged, and the bacterial pellet was kept at −70 °C until use. Cells were resuspended in 200 mL of lysis buffer [50 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 1.5 mM phenylmethanesulfonyl fluoride (PMSF)]. Then cells were broken by three cycles of sonication, and the resulting lysate was centrifuged. Solid ammonium sulfate [$(NH_4)_2SO_4$] was added to the turbid supernatant to 40% saturation; the mixture was stirred for 30 min and centrifuged. The supernatant was adjusted to 65% saturation

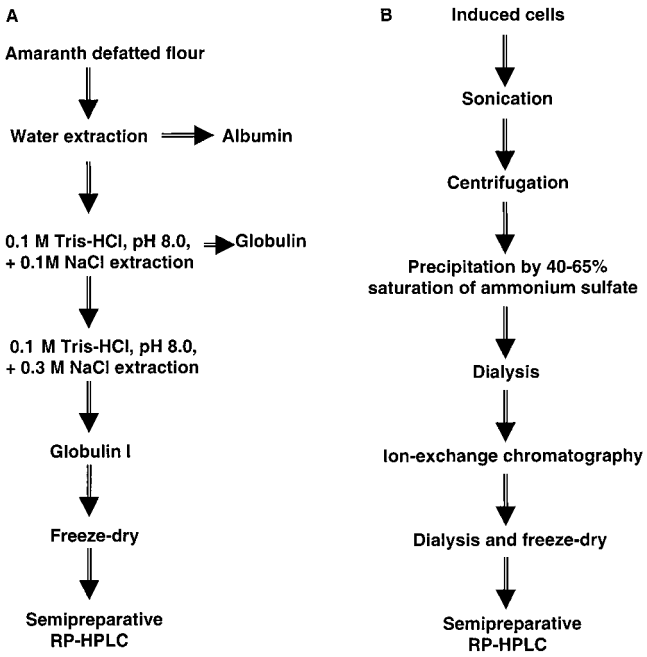

Figure 2. Purification procedures for both seed (A) and recombinant (B) amarantins.

with $(NH_4)_2SO_4$, stirred for 30 min, and centrifuged again. The precipitate containing the expressed 11S globulin was resuspended in buffer A (35 mM $K_2HPO_4 \cdot 3H_2O$, pH 7.6, 0.15 M NaCl, 10 mM 2-mercaptoethanol, and 0.02% NaN_3) and dialyzed overnight against buffer A with two changes. Urea was added to the dialyzed material to a final concentration of 6 M; the dialyzed protein was then applied to a 2 × 20 cm column of fast flow Q-Sepharose (Pharmacia Biotech), equilibrated with buffer B (buffer A plus 6 M urea), and eluted with a linear gradient of 0−0.5 M NaCl. The column effluent was monitored by OD_{280} and SDS-PAGE, and fractions containing recombinant amarantin were dialyzed and lyophilized. All steps were carried out at 4 °C, and centrifugations were for 20 min at 10000*g* at the same temperature (Kim et al., 1990, with some modifications). Finally, the freeze-dried sample was dissolved in buffer C (buffer A plus 3 M urea) and applied to a Vydac C_{18} semipreparative column (10 mm × 2.5 cm) for RP-HPLC (Figure 2). Elution was with a linear gradient of water containing 0.07% (v/v) trifluoroacetic acid (TFA) (A) and acetonitrile containing 0.05% (v/v) TFA (B), from 0% B for 4.6 min, then 0−50% B over 20 min, and then 50−0% B over 10 min. The fraction containing purified amarantin was freeze-dried (Buonocore et al., 1998).

Protein Determination. All protein concentrations were determined according to the method of Bradford (1976) with BSA as a standard.

In Vitro Refolding of Purified Recombinant Amarantin. The purified amarantin from *E. coli* was renatured in vitro using two methods: the dialysis method and the rapid dilution method according to Thompson et al. (1994). For the dialysis method the protein was dissolved at 1 mg/mL in 0.1 M Tris-HCl buffer, pH 8.0, containing 8 M urea, 0.1 mM dithiothreitol, 1 mM EDTA, and 1 mM PMSF and incubated at 20 °C for 2 h to allow for reduction. The pH was then adjusted to 3 by the addition of 0.1 M acetic acid and the solution diluted to 0.5 mg/mL before dialysis into 0.1 M acetic acid at 4 °C. The solution was then diluted to 0.1 mg/mL and dialyzed for 24 h against refolding buffer (0.1 M Tris-HCl, pH 8.0,1 mM EDTA, 0.5 M dimethylformamide, 2 M urea, 3 mM reduced glutathione, 0.3 mM oxidized glutathione, and 0.1 mM PMSF). The protein was then dialyzed and lyophilized. Samples for SDS-PAGE were taken before dialysis into 0.1 M acetic acid, and iodoacetamide was added to 100 mM to block free thiol groups to minimize rearrangements. In the rapid dilution method, the reduced protein in 0.1 M acetic acid was injected

directly into the refolding buffer to give a final concentration of 10 μg/mL. After 1 h of incubation at 20 °C, this solution was dialyzed against 0.1 M acetic acid at 4 °C and lyophilized. Samples for SDS-PAGE were taken and treated as described before.

Purification of Native Amarantin from Amaranth Seed. Defatted meal from mature seed was extracted sequentially (at 4 °C) according to the method of Chen and Paredes-López (1997) with some modifications: (a) defatted flour/water and albumin suspension was obtained; (b) pellet, 0.1 M Tris-HCl, pH 8.0, plus 0.1 M NaCl; (c) residue, 0.1 M Tris-HCl, pH 8.0, plus 0.3 M NaCl (buffer D) [in all extractions the flour/ or residue/solvent ratio was 1:10 (w/v)]. The supernatant (named globulin I and containing amarantin) was freeze-dried, dissolved in buffer D, and injected directly into semipreparative RP-HPLC using the same separating conditions as described above for the expressed amarantin (Figure 2).

RESULTS

Cloning of Amarantin. The amarantin cDNA encodes an amaranth 11S globulin subunit of 477 amino acid residues, including a 23 amino acid residue signal peptide. The mature protein has a molecular weight of 59.4 kDa and consists of an acidic polypeptide and a basic polypeptide with molecular weights of 35.4 and 24.0 kDa, respectively (Barba de la Rosa et al., 1996).

For amarantin expression in *E. coli*, the signal sequence was removed by PCR and replaced with an initiation codon (ATG). Then a 1.4 kb amarantin fragment generated by PCR was cloned into the *Nde*I and *Bam*HI restriction sites of the pT7-7 vector, which are located downstream from the T7 RNA polymerase-specific promoter with a strong ribosome binding site (rbs) (Figure 1).

Expression of Amarantin and Its Detection in *E. coli*. For a heterologous expression system to be of value, it should give high yields of protein that is correctly folded and processed. The choice of heterologous system therefore largely depends on the ease of handling of the host cell, the protein yield, and the ability to make the post-translational modifications necessary for protein function. *E. coli* was chosen in this study for a number of reasons. It multiplies rapidly and is easy to grow in large quantities. Thus, the construct pT7-AMAR was transformed into *E. coli* strain BL21 (DE-3), a widely used host for target gene expression that is inducible by the addition of IPTG. BL21 (DE-3) is an *E. coli* expression strain that has the advantage of being deficient in the *lon* protease and the Omp T outer membrane protease, both capable of degrading protein during purification (Grodberg and Dunn, 1988). Thus, at least some target proteins should be more stable in BL21 (DE-3) than in host strains containing these proteases.

Total cell proteins were extracted from recombinant bacteria before and after induction with IPTG, showing a new band in the induced samples at either 30 or 37 °C (Figure 3, tracks c−i). This new polypeptide had a molecular weight of ∼59 kDa, consistent with the expected size of the amarantin subunit encoded by its cDNA (Barba de la Rosa et al., 1996). The level of expression at 30 °C appeared to be greater at ∼6−9 h (tracks g and h, respectively) postinduction. At 37 °C, the highest amarantin expression was observed at an induction time of 6 h (track d). However, comparison between the sampling times at both growth temperatures showed that 6−9 h at 30 °C gave the highest

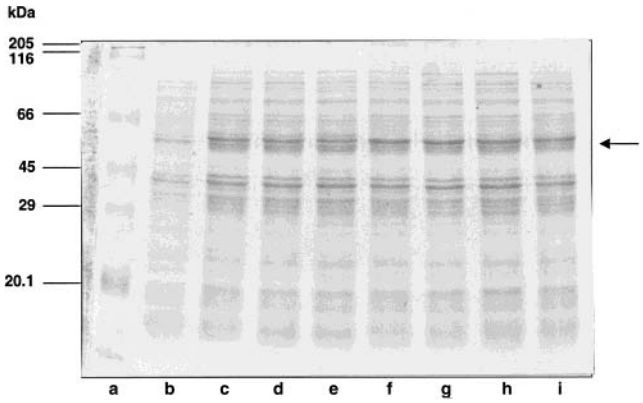

Figure 3. SDS-PAGE analysis of amarantin expression in *E. coli* at 37 °C (tracks c−e) and 30 °C (tracks f−i); (track a) molecular weight markers [myosin (205 kDa), β-galactosidase (116 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), and trypsin inhibitor (20.1 kDa)]; (track b) total cell proteins from *E. coli* before induction; (tracks c−e) total cell proteins from *E. coli* expressing amarantin at 3, 6, and 9 h after induction and 37 °C; (tracks f−i) total cell proteins from *E. coli* expressing amarantin at 3, 6, 9, and 12 h after induction and 30 °C. Each track contains a total protein of 20 μg. The arrow indicates amarantin band.

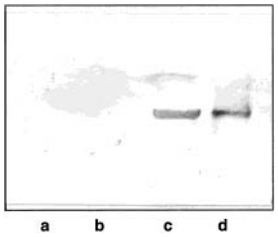

Figure 4. Immunoblot analysis of recombinant and seed amarantins using antiserum raised against amarantin isolated from amaranth grain: (track a) total cell proteins from *E. coli* containing pT7-7 vector without amarantin cDNA; (track b) total cell proteins from *E. coli* (containing pT7-AMAR vector) before induction; (track c) total cell proteins from *E. coli* expressing amarantin at 6 h after induction and 30 °C; (track d) amarantin subunit from amaranth seed (4 μg). As in Figure 3, 20 μg of total protein was loaded into tracks a−c.

expression. We therefore chose 6 h at 30 °C as our optimal expression conditions. A quantitative gel scanning by densitometry of the samples separated in tracks g and h (Figure 3) indicated that the yield of recombinant amarantin was ∼25−35 mg/L of culture. The identity of the expressed amarantin was confirmed by immunoblotting, which showed a single major band that reacted strongly with the amarantin antiserum (Figure 4, track c); a similar result was obtained with seed amarantin (track d), and western blot analysis also confirmed that the pT7-7 vector and uninduced cells contained no immunoreactive polypeptides (tracks a and b, respectively). We also measured the level of transgenic amarantin expression in *E. coli* using western blot assay; this analysis indicated that the recombinant amarantin yield was 32 mg/L of culture, which provides a more reliable estimate than that obtained by scan of total protein extract.

To determine the N-terminal amino acid sequence of the expressed protein, a sample of amarantin purified by ion-exchange chromatography and RP-HPLC (see below) was subjected to automated Edman degradation using an Applied Biosystems Procise sequencer. Only traces of amino acids were released from each cycle, indicating that the protein was probably N-terminally blocked.

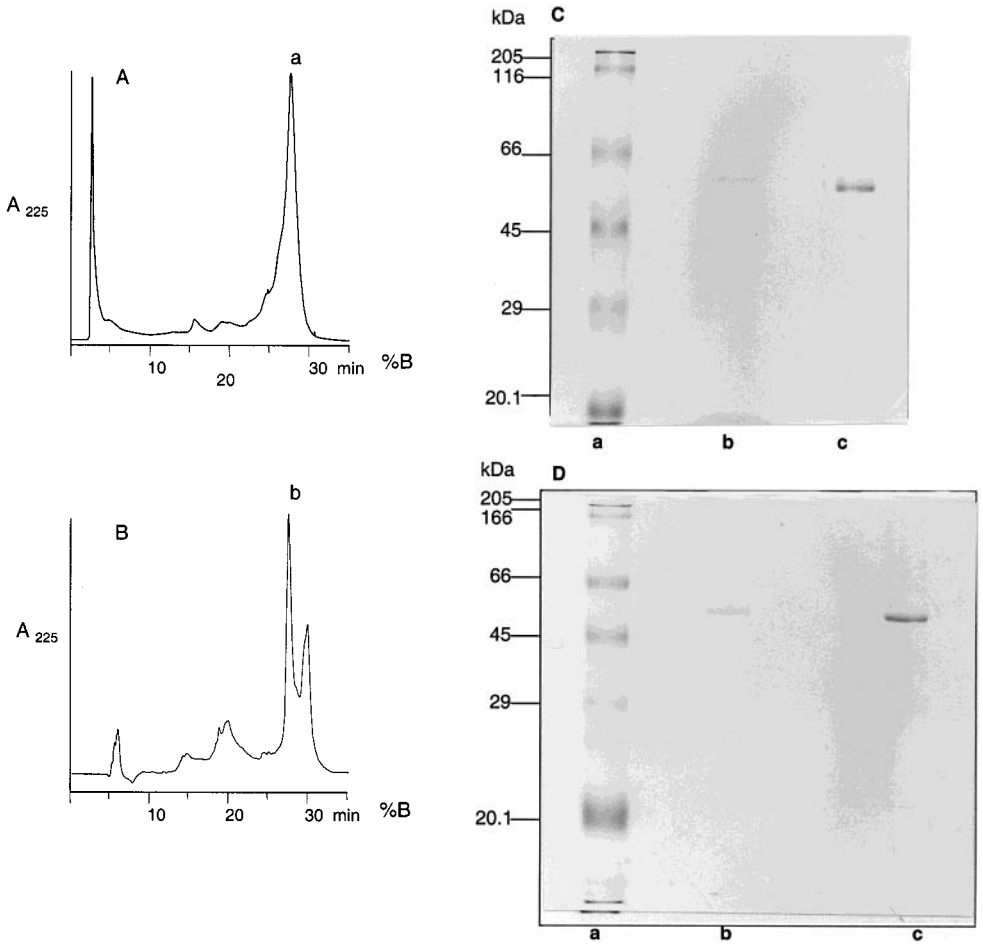

Figure 5. RP-HPLC of expressed and seed (native) amarantins, their SDS-PAGE, and in vitro refolding of recombinant amarantin: (A) expressed amarantin purified by semipreparative RP-HPLC (peak a); (B) native amarantin purified from amaranth seed globulin I fraction by RP-HPLC (peak b) (semipreparative RP-HPLC conditions: flow rate, 2.5 mL/min; temperature, 20 °C; absorbance at 225 nm); (C and D) SDS-PAGE of recombinant amarantin purified by semipreparative RP-HPLC corresponding to *E. coli* amarantin peak (with retention time of 27.9 min) of part A and refolded in vitro, respectively [tracks a, standard proteins described in Figure 3; tracks b, reducing conditions; tracks c, after refolding using the rapid dilution (part C) or dialysis procedures (part D)].

Purification of Amarantin from *E. coli.* The expressed amarantin was mostly precipitated at 40−65% saturation of $(NH_4)_2SO_4$ as judged by SDS-PAGE (results not shown). The precipitated protein was desalted by dialysis and then fractionated on a Q-Sepharose column and eluted with a linear saline gradient. However, this ion-exchange chromatography step, followed by cryoprecipitation, failed to purify the recombinant amarantin, in contrast to the reported purification of recombinant *E. coli* glycinin (soybean 11S globulin) (Kim et al., 1990). The expressed amarantin was present in only four fractions, although contaminating bacterial proteins were coeluted and subsequently precipitated during extensive dialysis against cryoprecipitation buffer (data not shown). Urea (6 M) was therefore used, which allowed us to separate the *E. coli* proteins, particularly those with higher molecular weights from amarantin; it is probably these proteins that showed strong noncovalent interactions with amarantin and were greatly reduced by urea. As a result the amarantin subunit was eluted as a single peak between 0.20 and 0.23 M NaCl, as expected on the basis of the properties of the seed globulin fraction containing amarantin, which similarly eluted at 0.20 M NaCl from DEAE-Sepharose CL-6B (Barba de la Rosa et al., 1992b). The electrophoretic profile of the Q-Sepharose peak revealed that the amarantin was obtained nearly pure, although a few contaminating proteins were still present (results not shown).

The amarantin was finally purified using semipreparative RP-HPLC (Figure 5A). The Q-Sepharose fractions containing amarantin were pooled and then dialyzed against distilled water overnight at 4 °C. The dialyzed proteins were then freeze-dried, redissolved in buffer A containing 3 M urea, and passed through a C_{18} semipreparative RP-HPLC column. The expressed amarantin gave a major peak eluting at ∼47% B and showing a retention time of 27.9 min (Figure 5A). This amarantin peak was collected, dialyzed, and freeze-dried, giving a single homogeneous band by SDS-PAGE (Figure 5C,D).

We also developed a fast procedure for the purification of native amarantin from seed also using RP-HPLC but eliminating the need of Sephacryl S-300 gel filtration chromatography as reported by Barba de la Rosa et al. (1996) and Chen and Paredes-López (1997). Briefly, the lyophilized globulin I fraction was dissolved in buffer D and then applied directly onto the RP-HPLC column (Figure 2). The seed amarantin also gave a major peak having the same retention time (27.9 min) as the protein purified from *E. coli* (Figure 5B). SDS-PAGE of this peak with reducing agent yielded three bands: an acidic

polypeptide of 36 kDa and a basic polypeptide of 24 kDa plus a polypeptide of ~59 kDa of unprocessed amarantins (data not shown), as previously reported by Barba de la Rosa et al. (1996) and Chen and Paredes-López (1997). These results suggest that the surface hydrophobicities of the seed and recombinant 11S globulins determined by RP-HPLC were very similar. Tamas et al. (1994) used a similar approach to demonstrate that C hordein, a barley prolamin expressed in *E. coli*, had a surface hydrophobicity similar to that of the native prolamin. Similarly, D'Ovidio et al. (1997) noted that a recombinant wheat prolamin (the high molecular weight glutenin subunit 1Dx5) produced in *E. coli* showed similar surface hydrophobicity as measured by RP-HPLC as the protein isolated from wheat seeds.

Although urea concentrations of 3 and 6 M were used in the current study, the RP-HPLC analyses indicate that it retained or regained some to most of its native conformation to exhibit surface hydrophobicity similar to that of the native protein. Nevertheless, we decided to refold the protein in vitro to ensure that it was correctly folded for future studies. We used either dialysis or rapid dilution into refolding buffer containing 2 M urea and 0.5 M dimethylformamide to maintain solubility and reduce subunit interactions and a mixture of reduced and oxidized glutathione as an oxido-shuffling system. The dialysis and rapid dilution methods gave essentially identical results (Figure 5C,D), the refolded protein migrating more quickly than the reduced protein, which was presumably due to the presence of intrachain disulfide bonds resulting in a more compact conformation. Such electrophoretic behavior has been well documented in the literature for other in vitro refolded proteins containing disulfide bridges, including both storage and nonstorage proteins (Hirose et al., 1989; Shani et al., 1992; Thompson et al., 1994; Buonocore et al., 1998).

DISCUSSION

We have obtained good level expression of an amarantin subunit in *E. coli*, which compares well with those reported for other 11S storage globulins using different *E. coli* host/vector combinations (Kim et al., 1990a; Tai et al., 1999). In the present research, the expressed protein was precipitated by the addition of $(NH_4)_2SO_4$ and then purified to homogeneity using ion-exchange chromatography followed by RP-HPLC. The recombinant globulin was readily identified at high levels in the bacterial extract, by SDS-PAGE and western blot analysis; in addition, its surface hydrophobicity determined by RP-HPLC was similar to that of 11S globulin isolated from amaranth grain. However, we were not able to confirm that the recombinant protein had the correct N-terminal sequence because it was apparently blocked. It is of interest that studies carried out with a range of other expressed or native storage proteins from seeds have shown the presence of blocked N termini, but their physicochemical, biochemical, and functional properties were apparently not affected (Tao and Kasarda, 1989; Thompson et al., 1994). The expressed amarantin was also refolded in vitro, and it is now necessary to confirm that the correct conformation has been adopted; we are therefore carrying out studies to compare the in vitro refolded and native proteins, determining their secondary and tertiary structures by circular dichroism, infrared and fluorescence spectroscopy, and small-angle X-ray scattering and their disulfide patterns, thermostability, and functional properties. The establishment of a system to produce high levels of correctly folded protein will allow us to systematically design and modify its structure to further improve its food quality as a prelude to overexpression in bioreactors or transgenic plants.

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LITERATURE CITED

Barba de la Rosa, A. P.; Gueguen, J.; Paredes-López, O.; Viroben, G. Fractionation procedures, electrophoretic characterization, and amino acid composition of amaranth seed proteins. *J. Agric. Food Chem*. **1992a**, *40*, 931−936.

Barba de la Rosa, A. P.; Paredes-López, O.; Gueguen, J. Characterization of amaranth globulins by ultracentrifugation, chromatographic techniques. *J. Agric. Food Chem*. **1992b**, *40*, 937−940.

Barba de la Rosa, A. P.; Herrera, A.; Utsumi, S.; Paredes-López, O. Molecular characterization, cloning and structural analysis of a cDNA encoding an amaranth globulin. *J. Plant Physiol*. **1996**, *149*, 527−532.

Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*. **1976**, *72*, 248−254.

Buonocore, F.; Bertini, L.; Ronchi, C.; Békés, F.; Caporale, C.; Lafiandra, D.; Gras, P.; Tatham, A. S.; Greenfield, J. A.; Halford, N. G.; Shewry, P. R. Expression and functional analysis of M_r 58 000 peptides derived from the repetitive domain of high molecular weight glutenin subunit 1Dx5. *J. Cereal Sci*. **1998**, *27*, 209−215.

Chatuverdi, A.; Sarojini, G.; Devi, N. L. Hypocholesterolemic effect of amaranth seeds (*Amaranthus exculantus*). *Plant Foods Hum. Nutr*. **1993**, *44*, 63−70.

Chen, S.; Paredes-López, O. Isolation and characterization of the 11S globulin from amaranth seeds. *J. Food Biochem*. **1997**, *21*, 53−65.

D'Ovidio, R.; Anderson, O. D.; Masci, J.; Skerritt, J.; Porceddu, E. Construction of novel wheat high-Mr glutenin subunit gene variability: Modification of the repetitive domain and expression in *E. coli*. *J. Cereal Sci*. **1997**, *25*, 1−8.

FAO/WHO. Protein quality evolution: Report of a joint FAO/WHO expert consultation. In *FAO Food and Nutrition Paper*; Food and Agriculture Organitization of the United Nations: Rome, Italy, 1991.

Fido, R.; Tatham, A. S.; Shewry, P. R. Western blotting analysis. In *Methods in Molecular Biology—Plant Gene Transfer and Expression Protocols*; Jones, H., Ed.; Humana Press: Totowa, NJ, 1995; Vol. 49, pp 423−437.

Grodberg, J.; Dunn, J. J. Ompt encodes the *Escherichia coli* outer membrane protease that cleaves T7-polymerase during purification. *J. Bacteriol*. **1988**, *170*, 1245−1253.

Guzmán-Maldonado, S. H.; Paredes-López, O. Functional products of indigenous plants to Latin America: Amaranth, quinoa, common beans and botanicals. In *Functional Foods*: *Biochemical and Processing Aspects*; Mazza, G., Ed.; Technomic Publishing: Lancaster, PA, 1998; Chapter 9, pp 293−328.

Hirose, M.; Akuta, T.; Takahashi, N. Renaturation of ovotransferrin under two-step conditions allowing primary folding of the fully reduced form and the subsequent regeneration of the intramolecular disulfides. *J. Biol. Chem*. **1989**, *264*, 16867−16872.

Kim, C. S.; Kamiya, S.; Kanamori, J.; Utsumi, S.; Kito, M. High-level expression purification and functional properties of soybean proglycinin from *Escherichia coli*. *Agric. Biol. Chem*. **1990**, *54*, 1543−1550.

Konishi, Y.; Yoshimoto, H. Amaranth globulins as a heat-stable emulsifying agent. *Agric. Biol. Chem.* **1989**, *53*, 3327−3328.

Konishi, Y.; Fumita, Y.; Ikeda, K.; Okuno, K.; Fuwa, H. Isolation and characterization of globulin from seeds of *Amaranthus hypochondriacus*. *Agric. Biol. Chem.* **1985**, *49*, 1453−1459.

Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680−685.

Lehmann, J. W. Case history of grain amaranth as an alternative crop. *Cereal Foods World* **1996**, *41*, 399−411.

Marcone, M. F. Evidence confirming the existence of a 7S globulin-like storage protein in *Amaranthus hypochondriacus* seed. *Food Chem.* **1999**, *65*, 533−542.

Marcone, M. F.; Yada, R. Y. Isolation, purification, and characterization of the oligomeric seed globulin from *Amaranthus hypochondriacus*. *Agric. Biol. Chem.* **1991**, *55*, 2281−2289.

Marcone, M. F.; Yada, R. Y. Study of the charge profile and covalent subunit-association of the oligomeric seed globulin from *Amaranthus hypochondriacus*. *J. Agric. Food Chem.* **1992**, *40*, 385−389.

Romero-Zepeda, H.; Paredes-López, O. Isolation and characterization of amarantin and the 11S amaranth seed globulin. *J. Food Biochem.* **1996**, *19*, 329−339.

Rooke, L.; Békés, F.; Fido, R.; Barro, F.; Gras, P.; Tatham, A. S.; Barcelo, P.; Lazzeri, P.; Shewry, P. R. Overexpression of a gluten protein in transgenic wheat results in greatly increased dough strength. *J. Cereal Sci.* **1999**, *30*, 115−120.

Segura-Nieto, M.; Barba de la Rosa, A. P.; Paredes-López, O. Biochemistry of amaranth proteins. In *Amaranth Biology, Chemistry and Technology*; Paredes-López, O., Ed.; CRC Press: Boca Raton, FL, 1994; Chapter 8, pp 76−95.

Segura-Nieto, M.; Shewry, P. R.; Paredes-López, O. Globulins of the pseudocereals: Amaranth, quinoa and buckwheat. In *Seed Proteins*; Shewry, P. R., Casey, R., Eds.; Kluwer Academic Publishers: Dordrecht, The Netherlands, 1999; Chapter 19, pp 453−475.

Shani, N.; Steffen-Campbell, J. D.; Anderson, O.; Greene, F.; Galilli, G. Role of the amino- and carboxi- terminal regions in the folding and oligomerization of wheat high molecular weight glutenin subunits. *Plant Physiol.* **1992**, *98*, 433−441.

Shewry, P. R. Manipulation of seed storage proteins. In *Transgenic Plant Research*; Lindsey, K., Ed.; Harwood Academic Publishers: Reading, U.K., 1998; Chapter 8, pp 135−149.

Shewry, P. R.; Napier, J. A.; Tatham, A. S. Seed storage proteins: Structure and biosynthesis. *Plant Cell* **1995**, *7*, 945−956.

Sindhu, A. S.; Zheng, Z.; Murai, N. The pea seed storage protein legumin was synthesized, processed, and accumulated stably in transgenic rice endosperm. *Plant Sci.* **1997**, *130*, 189−196.

Studier, F. W.; Rosenberg, A. H.; Dunn, J. J.; Dubendorff, J. W. Use of T7 polymerase to direct expression of cloned genes. *Methods Enzymol.* **1990**, 60−89.

Tai, S. S.; Wu, L. S.; Chen, E. C.; Tzen, J. T. Molecular cloning of 11S globulin and 2S albumin, the two major seed storage proteins in sesame. *J. Agric. Food Chem.* **1999**, *47*, 4932−4938.

Tamas, L.; Greenfield, J.; Halford, N. G.; Tatham, A. S.; Shewry, P. R. A β-turn rich barley seed protein is correctly folded in *Escherichia coli*. *Protein Express. Purif.* **1994**, *5*, 357−363.

Tao, H. P.; Kasarda, D. D. Two-dimensional gel mapping and N-terminal sequencing of LMW-glutenin subunits. *J. Exp. Bot.* **1989**, *40*, 1015−1020.

Thompson, S.; Bishop, D. H.; Madgwick, P.; Tatham, A. S.; Shewry, P. R. High-level expression of a wheat LMW glutenin subunit using a baculovirus system. *J. Agric. Food Chem.* **1994**, *42*, 426−431.

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