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Mature *Amaranthus hypochondriacus* seeds contain non-processed 11S precursors

María Isabel Molina, Ariana Circosta, María Cristina Añón, Silvana Petruccelli *

Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA), Facultad de Ciencias Exactas, Universidad Nacional de La Plata, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), calle 47 y 116, 1900- La Plata, Argentina

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Abstract

Amaranth is a dicotyledonous plant whose major seed storage proteins are globulins and glutelins. An unique feature of amaranth seeds is the presence of a fraction named albumin-2, that is extractable with water only after an exhaustive extraction of globulins and albumin-1. In this work, we tested the hypothesis that albumin-2 fraction could be constituted by a non-processed 11S globulin (proglobulin). To this end, the gene encoding the amaranth 11S subunit was cloned and expressed in *Escherichia coli*. Subsequently, the recombinant proglobulin and albumin-2 purified from seeds were treated with a sunflower vacuolar processing enzyme (VPE). A 55 kDa component of albumin-2 was specifically cleaved into 38 and 17–15 kDa polypeptides, as a consequence of this endoproteolytic cleavage a change of the oligomeric state from trimeric to hexameric was observed. Amaranth 11S globulin fraction was not modified under these proteolysis conditions. Using VPE-specific antibodies, it was shown that amaranth expresses a 57 kDa VPE, and that both developing and mature amaranth seeds have VPE activity, although the increase of this activity during amaranth seed development is higher than that observed for sunflower seeds. These results confirm the presence of unprocessed 11S precursors in mature amaranth seeds; this phenomenon cannot, however, be attributed to low VPE activity during developing of amaranth seeds.

Keywords: *Amaranthus hypochondriacus* L.; Amaranthaceae; Seed storage proteins; 11S globulins; Albumin-2; Recombinant amaranth globulin; Storage protein processing; Legumains

1. Introduction

Amaranthus genus is a non-grassy species, with good prospects to become a cereal-like grain crop, since it produces high yields of nutritious and edible seeds as well as having high agronomic potential (resistance to drought, heat and pests, and adaptability to inhospitable environments) (National Research Council, 1984; Bressani et al., 1987). Although frequently called as pseudo-cereal, amaranth is a dicotyledonous plant that produces starchy seeds with a protein content of 12–18% w/w, higher than cereals. Moreover, its nutritional value is remarkable based on its content of available lysine and sulfur amino acids, digestibility and protein utilization efficiency (Konishi et al., 1985; Bressani, 1994).

According to Osborne (1924), plant seed proteins can be classified into albumins, globulins and glutelins based on their solubility in water, saline, and NaOH solutions, respectively. Similar to other dicotyledonous plants, amaranth seeds contain albumins, globulins and glutelins, but lack the prolamins that are often present in cereals (Segura-Nieto et al., 1994; Muntz, 1998; Shewry and Halford, 2002). The major globulins of amaranth seeds are hexameric 11S legumin-like proteins, that are polymorphic and have lower solubility than soybean globulins (Barba De La Rosa et al., 1992a,b; Chen and Paredes-López, 1997; Martínez et al., 1997). Subunit composition of amaranth 11S globulin is very similar to that of glutelins (Abugoch et al., 2003), in agreement with the fact that both fractions belong to the same

* Corresponding author. Tel.: +54 221 4249287; fax: +54 221 425 4854.
E-mail address: silvana@biol.unlp.edu.ar (S. Petruccelli).

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structural gene family (Dunwell, 1998). In addition to globulins and glutelins, amaranth seeds contain a fraction named albumin-2, that represent 30% of total seed proteins and that is extractable with water only after exhaustive extraction of globulins and albumin-1 (Konishi et al., 1991). Albumin-2 is only found in *Amaranthus* species, and even closely related species as the pseudo-cereals quinoa (*Chenopodium quinoa*) and buckwheat (*Fagopyrum esculentum*) lack proteins with this distinctive solubility (Konishi et al., 1991; Nakamura et al., 1998). Although the structural components, physicochemical and immunochemical properties of the albumin-2 fraction, so-called globulin P, are similar to those of 11S globulins (Martínez et al., 1997; Castellani et al., 1999, 2000; Aphalo et al., 2004) the origin of this fraction is still unknown.

Precursor 11S globulins (preproproteins) are synthesized by rough endoplasmic reticulum. After removal of the signal sequence, the proglobulin subunits aggregate into trimers and move through the Golgi apparatus to the vacuoles, being processed by vacuolar processing enzymes (VPE) into the corresponding mature 11S hexameric forms (Dickinson et al., 1987; Jung et al., 1998; Adachi et al., 2001, 2003). Vacuolar processing enzymes belong to the asparaginyl-specific cysteine endopeptidase family known as legumains (Kembhavi et al., 1993), which, during seed development, hydrolyze the Asn-Gly peptide bond localized at the hypervariable region IV of 11S storage protein subunits. This specific cleavage is responsible for oligomerization of trimeric proglobulins into hexameric globulin (Nielsen, 1985; Nielsen et al., 1995; Jung et al., 1998; Adachi et al., 2001; Muntz et al., 2002). Although VPE participates in deposition and mobilization of storage globulins during seed maturation and germination/seedling growth, it has been shown that a knock-out of VPE is not lethal in *Arabidopsis thaliana* (Gruis et al., 2002). Seed asparaginyl endopeptidases have been studied in *A. thaliana*, jackbean; castor bean, soybean and *Vicia sativa* (Shimada et al., 1994; Hara-Nishimura et al., 1995; Hiraiwa et al., 1997; Okamoto and Minamikawa, 1998; Fischer et al., 2000; Muntz et al., 2002) but not in amaranth.

In this work, we investigated the hypothesis that amaranth albumin-2 is a non-processed 11S globulin or proglobulin. Using recombinant 11S proglobulin and albumin-2, purified from seeds, we prove that these proteins are processed into mature 11S forms by a sunflower seed VPE extract. In addition, we studied VPE activity in maturing and mature amaranth seeds to test the hypothesis that proglobulins accumulate in mature seeds as a consequence of low VPE activity in developing amaranth seeds.

2. Results and discussion

2.1. Amaranth storage protein isolation and study of the main components of the fractions

Amaranth seed proteins were isolated by sequential extraction with different solvents following the fractionation scheme shown in Fig. 1a (Konishi et al., 1991; Castellani et al., 1999). The globulin (G) fraction and albumin-2 (A-2) fraction were characterized by SDS-PAGE under non-reducing (NR) and reducing (R) conditions. The main components of the G-fraction were 60, 57, 55, 38, 31, 17 and 15 kDa proteins (Fig. 1b, NR, lane G). All these components, except the 60 kDa, belong to the 11S globulin family (Barba De La Rosa et al., 1992a,b; Segura-Nieto et al., 1992; Martínez and Añón, 1996; Martínez et al., 1997). The 55 and 57 kDa proteins (αβ subunits) of G-fractions were reduced by 2-mercaptoethanol with the simultaneous increase of 38 and 31 kDa α-polypeptides and 17 and 15 kDa β-polypeptides which is the behavior expected for processed 11S subunits (Fig. 1b, R, lane G). A minor component of the G-fraction was a 48 kDa that belongs to the 7S globulin family (Fig. 1b, R, lane G) (Marcone, 1999). The main components of A-2 fraction were 55 and 57 kDa proteins (Fig. 1b, NR, lane A-2). The 57 kDa protein was dissociated into α- and β-polypeptides by reduction with 2-mercaptoethanol, therefore it is also a processed 11S subunit (Fig. 1b, R, lane A-2). In contrast,

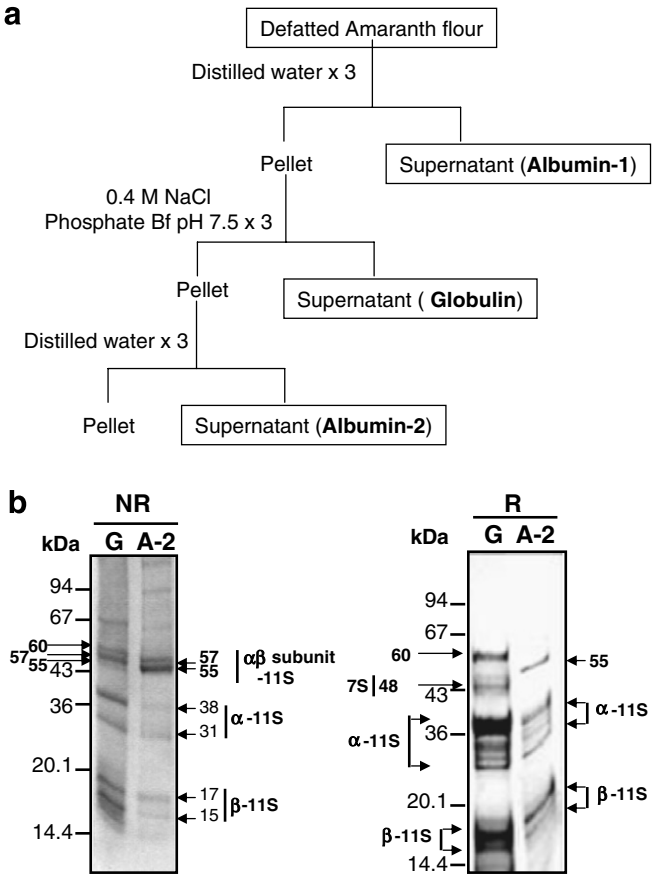

Fig. 1. (a) Schematic representation of the procedure used to isolate amaranth protein fractions. (b) Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE), 12.5% under non-reducing (NR) and 5–15% under reducing conditions (R) of globulin (lanes G) and albumin-2 (A-2) fractions stained with Coomassie Blue R-250. Arrows indicate the size of major components that are discussed in the text, including 7S and 11S globulin components. Molecular markers are given on the left in kDa.

the 55 kDa protein remained without modification under this condition (Fig. 1b, R, lane A-2), thus it could be a non-processed 11S precursor.

2.2. Cloning and expression of amaranth 11S globulin in Escherichia coli

In order to test the hypothesis that the 55 kDa amaranth A-2 protein is a non-processed 11S precursor, the full-length cDNA (1470 nucleotides) encoding for the amaranth 11S globulin (Barba De La Rosa et al., 1996) was amplified by RT-PCR using specific oligonucleotide primers and cloned into pSK. The construct was verified by sequencing analysis. The cloned amaranth 11S globulin (preproglobulin) (available in the GenBank under the Accession No. EF177470) has 487 amino acid residues, with a molecular weight of 55.06 kDa. The sequence encoding for the amaranth 11S globulin was analyzed using SignalIP server (http://www.cbs.dtu.dk/services/SignalP/) (Nielsen et al., 1997) to identify the secretory signal peptide. Then a new forward oligonucleotide primer was designed to place in frame the amaranth 11S globulin gene with the N-terminal six histidine tag (His-Tag) of pET28a. After amplification by PCR, the gene encoding the amaranth 11S proglobulin was subcloned into pET28a to obtain pET-AmhG-23 (Fig. 2a). The His-Tag amaranth 11S proglobulin fusion protein obtained had 487 amino acid residues including, besides the His-Tag, a thrombin cleavage site and T7-tag, with a molecular weight of 54.98 kDa. For protein expression, pET-AmhG-23 was introduced into *E. coli* BL21(DE3)pLysS. Initially, induction was performed for 4 h at 37 °C, yielding a high amount of a 55 kDa protein (Fig. 2b). This protein was specifically recognized by A-2-specific antibodies (Fig. 2c). These antibodies were obtained by immunization of rabbits with the A-2 fraction purified from seeds. They reacted strongly with a 56 kDa protein present in A-2 fraction and also 11S globulins present in amaranth G-fraction, thereby indicating that the two fractions are very closely related (Aphalo et al., 2004). The immunodetection of recombinant amaranth proglobulin demonstrated that this serum is also able to recognize non-processed 11S precursors.

Amaranth 11S subunit produced by induction at 37 °C accumulated as inclusion bodies, in agreement with results reported by Medina-Godoy et al. (2004). In order to obtain soluble amaranth recombinant protein, expression conditions were modified by lowering the temperature (30 °C until cells reach $OD_{600\,nm}$ 0.5–0.6, and 20 °C after IPTG was added) and by supplementing the LB broth with sorbitol, a non-metabolizable carbon source that slows down bacterial metabolism, thereby favoring protein folding (Lilie et al., 1998). Under these conditions, a small proportion of soluble amaranth protein was obtained and purified using Ni-affinity column. The purified recombinant amaranth 11S proglobulin was then analyzed by SDS-PAGE to confirm the integrity and purity (Fig. 2d). This purified protein was used in the subsequent experiments.

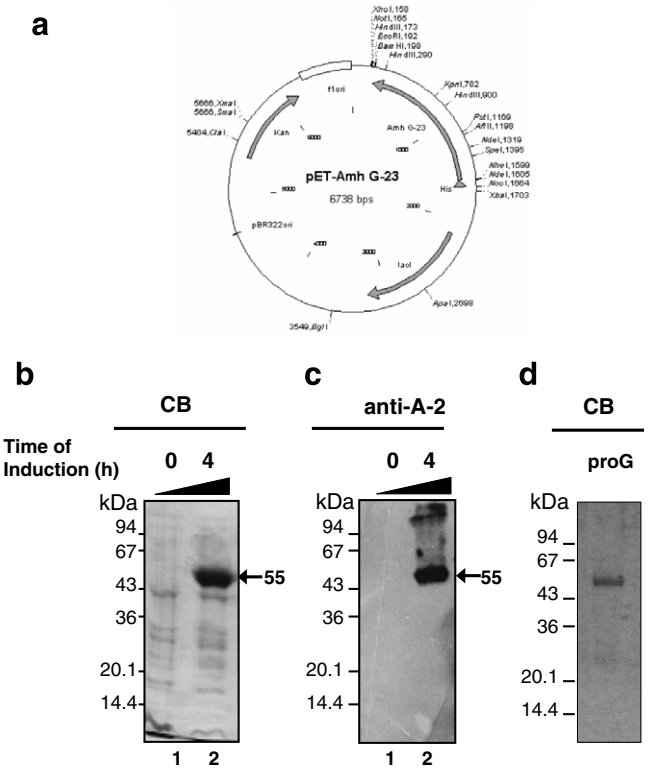

Fig. 2. (a) Amaranth 11S subunit expression vector. The cDNA without the signal peptide was fused to a six-histidine sequence, thrombin site and T7 tag at its 5′ end. (b,c) Analysis of total cell extracts of *E. coli* BL21 containing pETAmhG-23 before (lanes 1) and 4 h after induction with IPTG (lanes 2). (b) SDS-PAGE, 12.5% under reducing conditions, stained with Coomassie Blue R-250 (CB). (c) Immunoblot analysis with albumin-2-specific antibody (anti-A2). (d) Purified recombinant amaranth 11S proglobulin (proG). Arrows indicate unprocessed amaranth 11S subunit (proglobulin). Molecular markers are given on the left in kDa.

2.3. Processing of recombinant amaranth 11S globulin and seed purified A-2 by sunflower vacuolar processing enzyme extract

Storage protein 11S subunits are processed by vacuolar processing enzymes into α- and β-polypeptides that remain bound by disulfide bonds. As a result of this cleavage, two trimers interact to form hexamers (a schematic representation of this process is shown Fig. 3a). In order to study the processing of recombinant and seed-produced amaranth storage proteins, a sunflower VPE extract was prepared. Next, amaranth storage proteins were incubated with the VPE extract and separated by SDS-PAGE under reducing conditions to evaluate if the Asn-Gly peptide bond had been hydrolyzed, as shown in Fig. 3b. After 20 min of incubation with the sunflower VPE extract, the 55 kDa component of recombinant amaranth 11S proglobulin disappeared with the simultaneous appearance of a 38 kDa protein (α-polypeptide), two polypeptides of approximately 17 and 15 kDa (β-polypeptides) and a minor band of 31 kDa (Fig. 3b, lane 5 and 6, see black asterisk). The latter band can be attributed to a proteolytic cleavage in another Asn-containing bond. Seed purified

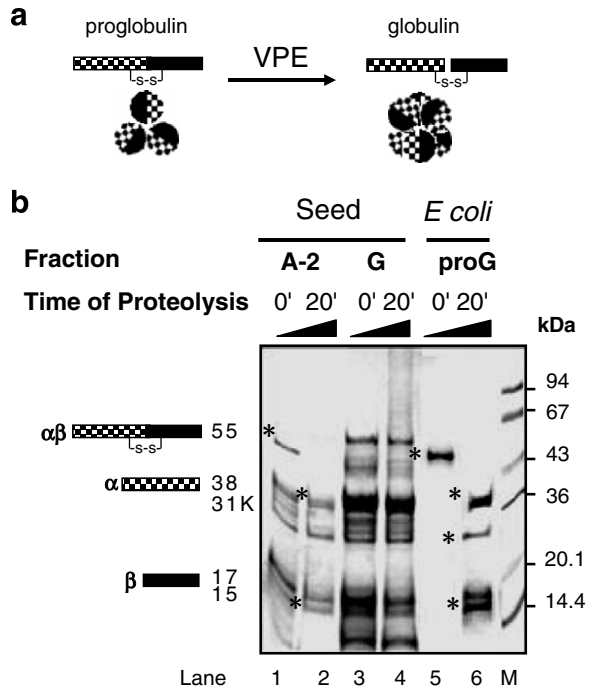

Fig. 3. Processing of amaranth proteins by the sunflower vacuolar processing enzyme (VPE). (a) Schematic representation of the processing of proglobulin by VPE. The proprotein is cleavage at Asn-Gly site to produce α- and β-polypeptides that remain bound by a disulfide bond. Trimeric proglobulin forms hexameric globulin after cleavage. (b) SDS-PAGE, 5–15% under reducing conditions of amaranth seed protein fractions and recombinant 11S proglobulin before (time 0) and 20 min after hydrolysis with VPE: Albumin-2 (A-2; lanes 1, 2); globulin 11S (G; lanes 3, 4), recombinant 11S subunit (proG, lanes 5, 6); lane M. Molecular markers (kDa) are indicated on the right.

A-2 fraction underwent changes similar to those of recombinant amaranth proglobulin: the 55 kDa disappeared (Fig. 3b, lanes 1 vs 2); and the 38, 31, 17 and 15 kDa polypeptides were constituted in the main components of the VPE-hydrolyzed A-2 fraction (Fig. 3b, lane 2). Under these proteolytic conditions, the amaranth G-fraction suffered only minor modification in the 48 kDa protein corresponding to 7S globulin, but the 11S globulin remained unaltered (Fig. 3b, lanes 3 and 4).

To corroborate that 11S globulins, present in the G-fraction, were not modified by VPE hydrolysis, seed protein fractions were also studied by immunoblot analysis with albumin-2 specific serum. Fig. 4 shows that albumin-2 55 kDa subunit and also the 38 and 17 kDa polypeptides were recognized by the serum (lane 1); after 20 min of VPE hydrolysis, the 55 kDa was completely processed. In contrast, no changes were observed in the amaranth G-fraction (Fig. 4, lanes 3 and 4). These results also show that although the albumin-2 polyclonal serum was obtained by rabbit immunization with seed purified A-2 fraction, it had strong reactivity against proteins present in G-fraction. These results demonstrate that the most important effect of VPE treatment is the proteolytic cleavage of major component of A-2 fraction: the 55 kDa subunit.

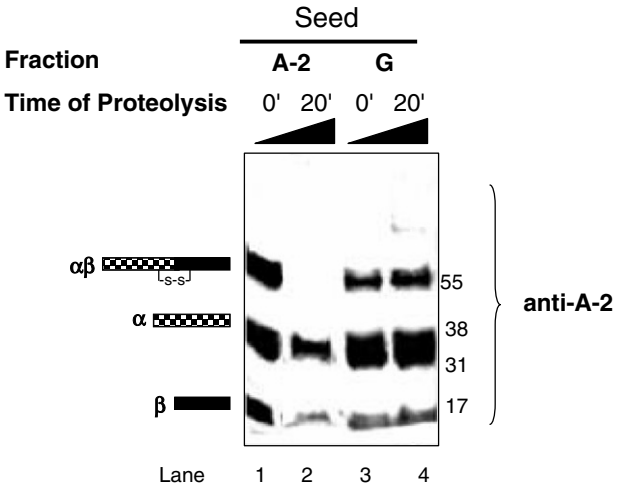

Fig. 4. Immunoblot analysis of amaranth seed fraction processed by sunflower vacuolar processing enzyme (VPE) extract. The A-2 fraction (lanes 1, 2) and G-fraction (lanes 3, 4) were treated during 0 and 20 min with VPE. After that, fractions were separated by SDS-PAGE under reducing conditions, blotted to nitrocellulose, and detected using albumin-2-specific antibodies. Molecular markers (kDa) are indicated on the right.

It is recognized that processing of 11S precursors by VPE is accompanied by transformation of trimeric proglobulins into hexameric globulins. To evaluate if the hydrolysis produced *in vitro* by sunflower VPE extract alters the oligomeric state, recombinant amaranth 11S proglobulins treated during 0 and 20 min with VPE, were analyzed by sedimentation in sucrose gradients. Fig. 5 shows that recombinant amaranth proglobulin is in a trimeric 7S form before VPE treatment, but turns into a hexameric 11S form after cleavage by VPE. Therefore recombinant amaranth proglobulin is processed into mature forms by sunflower VPE. However, *in vitro* proteolysis processing of amaranth proglobulin by sunflower VPE extract was not as precise as *in vivo* processing during seed maturation, besides in addition to the expected α- and β-polypeptides, minor bands also appeared. The fact that the cleavage produced the appearance of 11S forms supports our conclusion that the Asn-Gly peptide bond is hydrolyzed by this

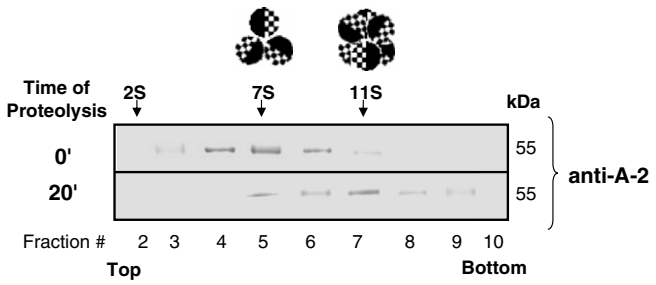

Fig. 5. Immunoblot analysis of recombinant 11S subunit in sucrose gradient fractions. The proG was treated during 0 and 20 min with VPE, and then the samples were fractionated in a sucrose linear gradient. After that, fractions were separated by SDS-PAGE under non-reducing conditions, blotted to nitrocellulose, and detected using albumin-2-specific antibodies.

enzyme. Processing of recombinant 11S proglobulin *in vitro* by VPE has also been reported by Medina-Godoy et al. (2004), but this is the first report that analyzed the processing of seed extracted protein fractions by VPE. Other proteolytic studies of the A-2 fraction, performed using trypsin and papain, showed that the non-processed 55 kDa subunit is not cleaved at the Asn-Gly peptide bond, indicating that this site is not hypersensitive to proteolytic attack (Aphalo et al., 2004). From these experiments, we conclude that both recombinant and albumin-2 amaranth proglobulins are processed into mature forms by sunflower VPE.

2.4. Vacuolar processing enzyme activity in developing and mature amaranth seeds

The existence of both processed 11S globulin (in the G-fraction) and unprocessed 11S proglobulin (in the A-2 fraction) in mature amaranth seeds may be a consequence of reduced VPE activity in developing amaranth seed compared with other seeds. In order to analyze this hypothesis, the presence of VPE and also its enzymatic activity were studied. In these experiments, developing and mature sunflower seeds were used as a reference. Total amaranth and sunflower seed extracts were prepared and analyzed using specific antibodies against soybean VPE. Fig. 6 shows that both sunflower and amaranth mature seeds have a 57 kDa protein that is recognized by antibodies specific against soybean VPE. This size is similar to that of other seed asparaginyl endopeptidases such as *A. thaliana* β-VPE, jackbean legumain; castor bean VPE and soybean VPE (Okamoto and Minamikawa, 1998).

VPE activity was measured using the fluorescent substrate Z-Ala-Ala-Asn-MCA, which is effectively cleaved by legumains but not by vignains, papain or cathepsin (Kembhavi et al., 1993; Okamoto and Minamikawa, 1998) (Table 1). The highest activity was observed in seed extracts at pH 5.5 in the presence of DTT, in accordance with known VPE characteristics (Hiraiwa et al., 1997; Okamoto and Minamikawa, 1998). No activity was observed in amaranth leaves. VPE activity in amaranth developing seeds was similar to that of maturing and mature sunflower seeds ($p < 0.05$), but lower than mature amaranth seeds (Table 1). Therefore the increase of VPE activity during seed maturation was higher for amaranth (4.5-fold) than for sunflower seeds. Based on this result, we discard the hypothesis that VPE are almost not active in amaranth developing seeds.

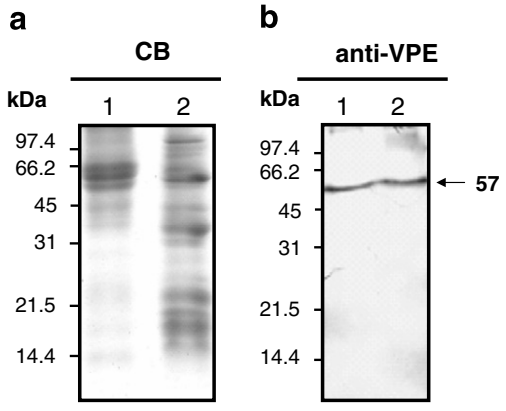

Fig. 6. Analysis of VPE in amaranth and sunflower extracts. SDS-PAGE 12.5% under non-reducing conditions stained with Coomassie Blue R-250 (a), immunoblot analysis with soybean VPE-specific antibodies (b). Lanes 1: sunflower seeds extract; Lanes 2: amaranth seeds extract. Arrows indicate the 57 kDa VPE. Molecular markers (kDa) are indicated on the left.

Table 1
VPE activity in maturing and mature amaranth seeds

Extract	Hydrolysis of Z-Ala-Ala-Asn-MCA (kat/g)			
	pH 5.5		pH 7.5	
	With DTT	Without DTT	With DTT	Without DTT
Developing amaranth seed	1.57 ± 0.18a	0.74 ± 0.06e	0.26 ± 0.04d	0.22 ± 0.06d
Mature amaranth seed	7.08 ± 0.17b	0.59 ± 0.07f	0.24 ± 0.05d	0.23 ± 0.05d
Developing sunflower seed	1.55 ± 0.20a	0.29 ± 0.05d	0.19 ± 0.02d	0.18 ± 0.05d
Mature sunflower seed	2.21 ± 0.12c	0.24 ± 0.05d	0.18 ± 0.04d	0.16 ± 0.04d
Amaranth leaf	0.22 ± 0.01d	0.19 ± 0.03d	0.14 ± 0.02d	0.12 ± 0.02d

Different letters indicate statistically significant difference ($p < 0.05$).

There are several plausible explanations for presence of non-processed globulins in mature amaranth seeds. One of them is differences in temporal expression patterns between VPE and the A-2 55 kDa subunit. Variations in the temporal expression pattern are observed for example in soybean developing seeds where 11S subunits are produced earlier than 7S subunits (Chen et al., 1989; Fujiwara and Beachy, 1994). In this context, the A-2 55 kDa subunit may be expressed during a seed development period different from other 11S subunits and VPE. Another possibility is that other factors such as accessibility to the substrate can limit the proteolysis of the A-2 fraction. It is well-known that this fraction has very low solubility and has a tendency to form aggregates, a characteristic that can limit the exposure of the Asn-Gly cleavage site (Konishi et al., 1991; Martínez et al., 1997; Castellani et al., 1998, 1999, 2000; Nakamura et al., 1998; Aphalo et al., 2004). Amaranth glutelins also contain the non-processed 55 kDa precursor (Abugoch et al., 2003; Aphalo et al., 2004). Finally, it is also feasible that a fraction of amaranth storage globulins accumulate inside in the endoplasmic reticulum of developing seeds and therefore remains in a compartment that lacks VPE as has been reported for accumulation of storage proteins in maize and rice (Herman and Larkins, 1999; Crofts et al., 2005).

3. Conclusions

This work has shown that the 55 kDa component of A-2 fraction is processed by sunflower VPE into 38 and 17–15 kDa polypeptides, in a similar way as for the recombinant

amaranth 11S proglobulin. This VPE processing of 11S proglobulin occurs with a concomitant change of the oligomeric state from trimeric to hexameric. In contrast, amaranth 11S G-fraction remains unmodified under these proteolysis conditions. These results corroborate the hypothesis that the A-2 fraction is a proglobulin. Moreover, we showed that although immature amaranth seeds have VPE activity, the increase of this activity during amaranth seed development is much higher than that in sunflower seeds, that may be an indication that this activity is not sufficient to complete process 11S proglobulins. As far as we know, amaranth is the only seed that accumulates 11S precursors. Our results cannot definitively explain the cause of the presence of the 55 kDa 11S precursor in mature amaranth seeds, and this question will be addressed in future investigations.

4. Experimental

4.1. Growth of plants

Amaranth (*Amaranthus hypochondriacus*, cultivar Mercado) and sunflower (*Helianthus* annuus cultivar ATAR TC 3003) were grown in a 1:1 (v/v) vermiculite–soil mixture at 25 °C in a greenhouse with a 16/8 h light/dark cycle. Maturing and mature seeds and leaf tissues were harvested from plants and used for total RNA and VPE isolation.

4.2. Amaranth storage protein isolation

Mature seeds were ground and the flour obtained was defatted using hexane (Aphalo et al., 2004). To obtain amaranth fractions with different solvent solubility, the fractionation scheme shown in Fig. 1 was followed. The defatted amaranth flour was successively treated three times with water to extract albumin, three times with 0.4 M NaCl, 32.5 mM K_2HPO_4, 2.6 mM KH_2PO_4 buffer pH 7.5 for globulin extraction (G), and three times with water to extract the albumin-2 (A-2) fraction as described (Castellani et al., 1999). The supernatants corresponding to globulins and albumin-2 were precipitated at pH 6 using 2 N HCl, and the pellets were then suspended in H_2O, neutralized with 0.1 N NaOH, and freeze-dried.

4.3. Preparation of amaranth and sunflower protein extracts containing vacuolar processing enzyme (VPE)

Protein extracts were prepared using maturing and mature amaranth and sunflower seeds, and also amaranth leaves. Developing sunflower seeds were classified according to size; 5–15 mg embryos were used as starting material for both VPE extraction and activity measure (Molina et al., 2006). Developing amaranth seeds were separated from mature seeds based on the appearance of their seed coat. Leaves and seeds were ground to a powder in liq. N_2, and proteins were extracted with 200 mM sodium phosphate buffer pH 7.5, 1.5 mM phenylmethanesulfonyl fluoride (PMSF), 1 mM EDTA. The resulting suspension was filtered through four layers of Miracloth (Calbiochem-Novabiochem), and centrifuged at 10,000*g* for 15 min at 4 °C. The supernatant from this step was stored at −80 °C until VPE activity was measured. Sunflower VPE protein extracts, used for proteolysis of amaranth storage proteins, were concentrated by addition of $(NH_4)_2SO_4$ to 80% of saturation. After 60 min, centrifugation (10,000*g*) for 30 min yielded a pellet that was dissolved in 20 mM sodium phosphate buffer, pH 7.5, 1 mM EDTA. The solution was dialyzed overnight, and then stored at −80 °C (Kembhavi et al., 1993).

4.4. cDNA cloning and constructs for expression in E. coli

Total RNA was prepared from *A. hypochondriacus* developing seeds using Trizol (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. A full-length 11S globulin cDNA (GenBank Accession No. X82121) was synthesized using 1 μg of total RNA with Superscript II-RNase H^- reverse transcriptase (Invitrogen) followed by amplification by PCR using the oligonucleotides 5′Amh (CTAG*TCTAGA*ATGGCTAAGTCTACTAATT, *Xba*I site underlined) and 3′Amh (CGCG*GGATCC*TTAGG-CAATGCTGATTTTCCTTCGGT, *Bam*HI site underlined). The PCR product was digested with *Xba*I and *Bam*HI and inserted into the plasmid pSK (Stratagene) to obtain pSK-Amh.

To construct the plasmid for expression of the amaranth 11S globulin in *E. coli* the coding sequence was amplified with the oligonucleotides 5′A-23 CCCA*GCTAGC*-GAAGGAAGGTTTAGAGAG, *NheI* sites underlined) and 3′Amh; the first primer was designed to remove the N-terminal signal peptide (23 amino acids). The PCR product was digested with *NheI* and *Bam*HI and was cloned into the expression vector pET28a (Novagen, Madison, WI) to obtain pET-AmhG-23. All constructs were verified by sequencing analysis.

4.5. Production of amaranth 11S globulin in E.coli

pET-Amh G-23 was transformed into *E. coli* strain BL21(DE3)pLysS for protein expression. Cells were grown at 30 °C in Luria–Bertani (LB) broth containing 0.66 M sorbitol, until cell density (OD_{600}) reached 0.5–0.6, and protein expression was induced by addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside for 15 h at 20 °C. Cells were harvested by spinning at 5,000xg for 4 min, and the cell pellets were resuspended in lysis buffer (20 mM Tris–HCl, pH 7.5, 500 mM NaCl, 0.1% (v/v) NP-40, 1 mM PMSF, 1 mg/ml lysozyme, 10 μg/ml DNAase and 10 μg/ml RNase). Lysis was completed using a sonicator. His-tagged proteins were purified under non-denaturing

conditions according to procedures provided by Novagen. The proteins were stored at −80 °C.

4.6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis

SDS-PAGE were performed according to Laemmli (1970) on linear gradient gels from 5% to 15% acrylamide or continuous 12.5% acrylamide gels using minislabs (Bio-Rad Mini Protean II model). After electrophoresis, gels were fixed and stained with Coomasie Brillant Blue R 250 or wet-blotted onto nitrocellulose membranes (Schleicher & Schuell Bioscience Inc., USA) according to Towbin (1979). Blots were blocked for 1 h in 3% non-fat dry milk in sodium phosphate, pH 7.4, 150 mM NaCl and then incubated with rabbit-specific antibodies against amaranth albumin-2 or soybean VPE (1:1000 dilution) at 37 °C for 1 h. Subsequently, the membranes were incubated with HRP conjugated anti-rabbit secondary antibody (Bio-Rad Laboratories, CA). Immunoreactive signals were detected with 4-chloro-1 naphthol or with luminol chemiluminescent substrate (Schneppenheim et al., 1991) and exposure to X-ray film.

4.7. Fractionation of proteins in sucrose gradients

Recombinant 11S proglobulin, with and without treatment with VPE, was loaded on a 10–30% (w/w) sucrose linear gradients, and centrifugation was performed at 50,000 rpm for 5 h at 10 °C in an Optima TL, Beckman Ultracentrifuge, using the TLS-55 swinging bucket rotor as described previously (Molina et al., 2004). The gradients were carefully fractionated in 150 μL aliquots starting from the top of the tube for analysis by Western blotting. Standard proteins, soybean 7S and 11S purified globulins were analyzed in separate tubes.

4.8. Assay of VPE activity

VPE activity (asparaginyl-specific cysteine endopeptidase, (EC 3.4.22.34) was measured using a fluorescent VPE-specific substrate: Z-Ala-Ala-Asn-MCA (Carbobenzoxy-L-Alanyl-L-Alanyl-L-Asparagine 4-Methyl-Coumaryl-7-Amide) (Peptide Institute Inc., Louisville, KY, USA) essentially as described by Kembhavi et al. (1993). Briefly, seed or leaf extracts were incubated in 100 mM sodium acetate pH 5.5, 0.02% PMSF, 5 mM EDTA (with and without 100 mM DTT) or 100 mM sodium phosphate pH 7.5, 0.02% PMSF, 5 mM EDTA (with and without 100 mM DTT) at 37 °C for 10 min, then Z-Ala-Ala-Asn-MCA working solution was added (10 μM in the reaction tube) and then fluorescence ($\lambda_{ex} = 355$ nm and $\lambda_{em} = 460$ nm) was measured at different times (0–60 min) using a microplate reader. Activities were expressed in katals (kat). Specific activities were calculated with reference to protein concentration, which was determined by the method of Bradford (1976), using bovine serum albumin (Sigma, St. Louis, MO) as a protein standard.

4.9. Statistical analysis

Two independent VPE protein extractions were performed and VPE activity of each sample was measured at least in triplicate. The results so obtained were statistically evaluated by variance analysis (ANOVA). The comparison of means was done by the Student–Newman–Keuls Method, with a level of significance (α) of 0.05. Both analyses were carried out using the SigmaStat, version 2.0, software (Jandel Corporation).

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