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Analysis of Globulin Maturation in Developing Sunflower Seeds

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Abstract. The synthesis of major storage globulin polypeptides has been examined in developing seeds of sunflower (*Helianthus annuus* L.). Analyses of total proteins and purified globulins, also called helianthinin, by gel electrophoresis and immunoelectrophoresis have shown that a burst of protein synthesis and accumulation occurs around 10 d after flowering. There is no mature globulin before that time and only small amounts of precursor forms can be detected. Thus, 10–12 d after flowering appears to be a transition period during which genetic information for the globulin becomes actively expressed. Immunoelectrophoresis has confirmed that globulin is the main storage protein, at seed maturation, accounting up to 70 % of total proteins per kernel. Pulse chase experiments have shown that synthesis initially involves the formation of high molecular mass precursors and that storage proteins are post-translationally processed. Intermediary products, with molecular mass higher than early translational products, can be detected, together with mature globulin polypeptides.

Helianthinin is the predominant protein species in sunflower seed (Gheyasuddin *et al.* 1970, Sabir *et al.* 1973, Baudet and Mossé 1977) and corresponds to a 11-S globulin with a high molecular mass, 320 kDa, comprising numerous polypeptides (Sabir *et al.* 1973, Ferjani and Ledoigt 1987, Ferjani 1988, This *et al.* 1988). The major helianthinin subunits, consisting of six acidic (α) and six basic (β) polypeptides are linked together by disulfide bond and make up an hexameric structure (Ferjani 1988). Storage proteins are synthesized on and translocated across the rough endoplasmic reticulum membrane. Eventually, they become deposited in distinct cellular vesicles, protein bodies, that most probably are derived from the endoplasmic reticulum (Larkins and Hurkman 1978; Ferjani *et al.* 1989). In sunflower, we have shown that 4 precursor polypeptides are synthesized on membrane-bound polysomes (Ferjani *et al.* 1989).

Biosynthesis of storage proteins is well-documented for legumin (Millerd *et al.* 1978, Sun *et al.* 1978, Croy *et al.* 1980; Spencer and Higgins 1980; Gifford

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and Bewley 1984), glycinin in soybean (Sengupta *et al.* 1981, Turmer *et al.* 1981, Barton *et al.* 1982) and globulins in *Cucurbitaceae* (O'Kennedy *et al.* 1979) and oat (Brinegar and Peterson 1982). Studies have been performed to characterize sunflower seed proteins (Sabir *et al.* 1973, Dalgalarrondo *et al.* 1984; Allen *et al.* 1985, This *et al.* 1988) but physiological and cytological aspects of their biosynthesis during sunflower seed development need to be resolved. The aim of the present work is to gain a better understanding of the biosynthesis and maturation process of helianthinin during the seed development. We report evidence showing that this protein is post-translationally processed from precursor forms with high molecular mass intermediary proteins, during early seed development.

MATERIAL AND METHODS

Plant material

Sunflowers (*Helianthus annuus* cv. Bolero) were grown under field conditions. Developing seeds were harvested at different stages, husked and immediately used or stored at – 80 °C.

Protein extraction

Seeds were crushed in a mortar with 50 mM Tris-HCl buffer (pH 8), 1 M NaCl, then centrifuged for 10 min at 15 000 rpm (RC2 B sorvall rotor SS 34) and the supernatant containing the soluble proteins was removed and conserved.

Pulse-labelling with ^{14}C amino-acids

Seeds harvested from different developing stages were husked and put into a mixture of labelled amino-acids (185×10^7 Bq/milliatom of carbon, CEA-France 2.96×10^4 Bq/seed) at 20 °C. Following the incubation period, soluble proteins were extracted and associated radioactivity was measured in TCA insoluble samples.

Pulse and chase experiments

Developing seeds were harvested and proteins were labelled by incubating husked grains at 16 daf in the mixture of ^{14}C amino acids for 3 h. The kernels were then washed in sterile distilled water (pH 7) and put into sterile distilled

Abbreviations : daf – days after flowering, PAGE – polyacrylamide gel electrophoresis, RER – rough endoplasmic reticulum, SDS – sodium dodecyl sulfate, TCA – trichloroacetic acid, Tris – tris(hydroxymethyl) aminomethane.

water for different periods. Then, kernels were quickly frozen in liquid nitrogen and soluble proteins were extracted in 50 mM Tris-HCl buffer (pH 8) 1 M NaCl.

Polyacrylamide gel electrophoresis and fluorography

SDS-PAGE was performed according to Laemmli (1970) with 12 % or 15 % acrylamide. Polypeptides were stained by Coomassie Blue. Gels for fluorography were treated with 1 M sodium salicylate (Chamberlain 1979) after destaining and were dried under vacuum on a slab gel drier. Fluorographs were obtained by exposure at – 70 °C to X-ray films (Agfa Gevaert curix RP1). The molecular mass of polypeptides was given by comigrating polypeptide markers (Pharmacia).

Immunoblots

Gels for Western blots were electrophoretically transferred onto nitrocellulose membranes (Schleicher and Schuell) using a transblot apparatus (Biorad). Polypeptides were then treated by globulin antibodies (Ferjani *et al.* 1989) and revealed with a Biorad Kit.

Immunoelectrophoresis

Soluble protein fractions (25 µg) were separated by electrophoresis in agarose gel comprising 1.2 % agarose (Sigma) in buffer 0.165 M Tris-HCl (pH 8.6), 0.4 M glycine, 0.02 % NaN_3 supplemented with 400 µg ml^{-1} globulin antibodies (40 mA for 18 h). The gels were then stained by 0.5 % Coomassie Blue and destained in a mixture of ethanol-acetic acid-distilled water (Laurell 1966).

RESULTS

Immunoblot analysis during sunflower seed development

Soluble polypeptides were separated by SDS-PAGE and a Western blot analysis was performed using mature helianthinin antibodies. The antihelianthinin serum had previously been obtained with purified globulin only containing polypeptides 30 kDa, 24 kDa and 22 kDa. During seed development, several polypeptide groups were selectively stained on immunoblots (Fig. 1).

A 52 kDa polypeptide was the primary stained polypeptide at the first stage (9 daf) and persisted throughout seed development (Fig. 1, arrow b). Mature globulin polypeptides (30 and 22–24 kDa) appeared from 12 daf and their staining was enhanced during seed development (Fig. 1, arrows d, e and f).

Other polypeptides are stained later. Some of these polypeptides were not conserved in mature grains as, for instance, 58–68 kDa polypeptides which are

present from 16 daf to 30 daf, (Fig. 1, arrow a) and polypeptides around 85 kDa which are stained from 23 to 30 daf. Other bands appeared during seed development and were present in mature kernel, as for instance three polypeptides around 40 kDa which are stained from 16 daf but were barely stained before (Fig. 1, arrow c) and two polypeptides 28–29 kDa which were stained from 19 daf (Fig. 1).

In vivo protein synthesis during seed development

For pulse-label studies, cotyledons were harvested from 5 to 14 daf and incubated for 3 hours with ^{14}C labelled aminoacids. Soluble proteins were analyzed by SDS-PAGE and fluorography. From 5 to 10 daf, mainly high Mm polypeptides were labelled (70 to 80 kDa) (Fig. 2).

Several polypeptides with lower Mm (14 to 30 kDa) were highly labelled from 12 daf. Among these labelled polypeptides, mature globulin polypeptides (30, 24 and 22 kDa) could be observed. They could not be detected before 12 daf.

For pulse and chase studies, 16 daf seeds were incubated for 3 hours with a ^{14}C aminoacid mixture and labelled soluble polypeptides were analyzed following different periods of chase in medium free of labelled aminoacids (Fig. 3). Up to 1 hour of chase, high Mm polypeptides (52 and 58–60 kDa) were strongly labelled. Other polypeptides (15, 22, 24 and 30 kDa) were also detected. After 2 hours of chase, lower Mm (36, 30, 24 and 22 kDa) labelled polypeptides appeared and their labelling increased for longer chase periods. For the same period, also, after 2 hours of chase several high Mm labelled polypeptides were decreasing relative to 1 h. With 24 h chase period, at least 4 labelled polypeptides (80, 75, 68 and 52 kDa) disappeared (Fig. 3). The 4–6 h chase periods mainly revealed the synthesis of very high Mm polypeptides (74 to 90 kDa).

Immunological analysis of globulin accumulation during seed development

Globulin polypeptides were quantified by rocket immunoelectrophoresis. In the soluble protein fraction, globulin polypeptides increased from 10 to 30 daf at about 0.45 mg per grain and per day. The amount increased from 0.2 mg to 7.2 mg per grain, representing from 5 to 72 % of total seed soluble proteins at maturity (Fig. 4).

DISCUSSION

Globulin represents the major component of the storage proteins of sunflower seed (Gheyasuddin *et al.* 1970; Sosulski and Fleming 1977). Several studies have concerned the biochemical composition and structure of sunflower proteins

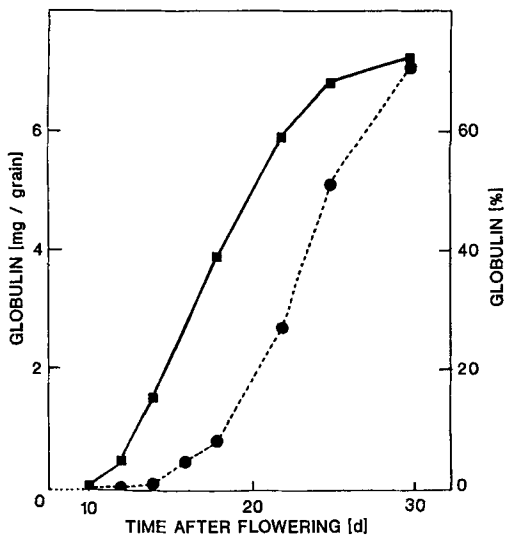

Fig. 4. Time course of globulin content in developing sunflower seeds, in mg per grain (dotted line) and in percentage of total soluble polypeptides (full line).

(Joubert 1955, Sabir *et al.* 1973, Baudet and Mossé 1977, Plietz *et al.* 1978, Schwenke *et al.* 1979, Reichelt *et al.* 1980; Dalgalarrondo *et al.* 1984), especially, in mature seeds.

Mature globulin biosynthesis begins for sunflower seed, with a lag period after flowering as it was previously shown in rice (Yamagata *et al.* 1982; Yamagata and Tanaka, 1986), soybean (Hill and Breidenbach 1974), sorghum (Subramanian *et al.* 1983), wheat (Greene 1983), *Ricinus* (Gifford *et al.* 1984), fenugreek (Sauvaire *et al.* 1984), bean (Sun *et al.* 1978) and pea (Millerd *et al.* 1978).

Polyacrylamide gel electrophoresis of soluble proteins has shown that in sunflower primary polypeptide biosynthesis is not related to globulin. Bewley and Black (1978) have suggested that the primary polypeptides are involved in non-storage processes occurring in the first stages of seed development.

Immunoelectrophoretic and SDS-PAGE analyses of total soluble proteins showed that globulin genes were not expressed till 8–9 daf. These results confirmed the observations previously reported (Allen *et al.* 1985, This *et al.* 1988). Using a helianthinin cDNA clone, they detected no helianthinin mRNA before 10 daf.

From 12 daf, protein synthesis increased in grains and especially that of globulin which rose from 10 % to 72 % total proteins between 13 and 30 daf. Therefore, as most storage proteins in dicotyledonous seeds (Hill and Breidenbach 1974, Millerd *et al.* 1978; Gifford *et al.* 1984), sunflower globulin is

synthesized during a specific period of seed development, after the highest level of corresponding mRNAs is reached.

The level of globulin, namely 72 % total proteins at 30 daf as measured by immunoelectrophoresis, is similar to that found for mature seeds of sunflower (Sabir *et al.* 1973, Baudet and Mossé 1977, Sosulski and Fleming 1977, Schwenke *et al.* 1979). Mature globulin polypeptides (22, 24 and 30 kDa) detected from 12 daf derive from high molecular mass polypeptides (75, 68, 54 and 52 kDa) which are related to early precursor forms as shown by immunoprecipitation of cell-free translation products (Ferjani *et al.* 1989). Sunflower globulin biosynthesis occurs on RER and protein body membranes on which ribosomal particles can be seen (Ferjani *et al.* 1989). Precursor forms are then processed into mature globulin polypeptides.

The general biosynthetic mechanism of 11-S globulin involves precursor forms having an acidic polypeptide component (α) and a basic polypeptide component (β) (Hara-Nishimura *et al.* 1985, Müntz *et al.* 1985; Pernollet 1985). The processing of globulin precursors into mature polypeptides involves several features peculiar to the biosynthesis of 11-S globulin, namely, S-S formation, assembly and posttranslational modifications. In sunflower, it was reported that HA10, a 12-S storage protein gene, has been completely sequenced and the only highly conserved region is that encoding the α/β cleavage site (Allen *et al.* 1985; Casey and Domoney 1987, Van der Haar *et al.* 1988). Thus, although the mature helianthinin structure was more complex than other 11-S globulins (Ferjani 1988), the biosynthetic scheme of helianthinin would be quite identical to the major 11-S storage proteins.

Higher molecular mass polypeptides (around 75–90 kDa) are transiently observed during sunflower seed development. The polypeptides, identified as globulin-like components on Western blot analysis, could be related to the mature polypeptide arrangement in protein body structure or to primary translation products which must be post-transcriptionally processed to form high Mm polypeptides as described for maize globulin (Kriz and Schwartz 1986). Developmental occurence of high molecular mass globulin-related polypeptides (up to 75 kDa) can represent intermediary precursors which are post-translationally modified following their integration into protein bodies. This processing can be inferred from the time-lag between the patterns of mRNA and globulin accumulations (Allen *et al.* 1985).

Smaller Mm polypeptides (38–44 kDa) which were previously described as mature globulin polypeptide species (Dalgalarrondo *et al.* 1984; This *et al.* 1988) appear to be the association of mature polypeptides since they are recognized by antibodies obtained from purified globulin made of 30, 24 and 22 kDa polypeptides and since they disappear from purified globulin (Ferjani et Ledoigt 1987). We have here observed that during seed development, 38–44 kDa polypeptides were stained on Western blots, when we used antibodies against

helianthinin (*i.e.*: 30, 24 and 22 kDa polypeptides). Therefore, the 38–44 kDa polypeptides appear to be modified forms of mature globulin in sunflower. Posttranslational modifications are often described for storage proteins such as legumin (Croy *et al.* 1980, Spencer and Higgins 1980), glycinin (Sengupta *et al.* 1981, Barton *et al.* 1982) and globulin (O'Kennedy *et al.* 1979, Brinegar and Peterson 1982). The present work has revealed such a sequence for the synthesis of these polypeptide forms during sunflower seed development.

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Figs. 1–3 at the end of the issue.

BOOK REVIEW

Marten, G. C., Matches, A. G., Barnes, R. F., Brougham, R. W., Clements, R. J., Sheath, G. W. (ed.): Persistence of Forage Legumes. – ASA, CSSA, SSSSA, Madison 1989. 552 pp. Hardcover US $ 19.00 + 1.9 outside the U.S.A.

This publication is a collection of papers from the proceedings of a trilateral workshop, held in Honolulu, Hawaii, 8–22 July 1988. This workshop was organized under the auspices and support of the Australian-United States of America Agreement of Scientific and Technical Cooperation and the New Zealand-United States of America Science and Technology Agreement.

The papers are divided by the theme into the following six chapters: Overview of problems with legumes, development and growth characteristics of legumes, major edaphic and climatic stresses, cultural practices and plant competition, plant-animal interface, major pests and diseases, and genetics and breeding for persistence. The authors of these papers solve the problems of persistence of forage legumes from the view of the conditions in the U.S.A., New Zealand and Australia and that is why they describe species of both tropical and temperate zones. Each of the papers is supplied with numerous references and the discussions of the participants of the proceedings. The publication also contains the problems solved in collaborative research of the participating countries with the names of the research workers and their work places. The book is a very interesting illustration of problems in the field of growing of forage legumes studied in the world and so can be useful to plant physiological specialists, and to those engaged in keeping techniques, plant protection, genetics and breeding of forage crops.

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