Synthesis and processing of thionin precursors in developing endosperm from barley (*Hordeum vulgare* L.)

F.Ponz, J.Paz-Ares, C.Hernández-Lucas, P.Carbonero and F.García-Olmedo

Departamento de Bioquímica, E.T.S. Ingenieros Agrónomos-UPM, Madrid-3, Spain

Communicated by E.Viñuela
Received on 23 February 1983; revised on 18 April 1983

Thionin is a lysine-rich polypeptide (mol. wt. 5000) which is synthesized in developing barley endosperm from ~8 days to ~30 days after anthesis. Two thionin precursors (THP1 and THP2) have been identified using monospecific antibodies (A-TH) prepared against the mature protein. THP1, which is the only polypeptide recognized *in vitro* by A-TH, is encoded by a 7.5S mRNA obtained from membrane-bound polysomes, and its alkylated derivative has an apparent mol. wt. of 17 800. THP2, which is selected together with mature thionin by A-TH among labelled proteins *in vivo*, differs from THP1 in apparent mol. wt. (17 400 alkylated) and in electrophoretic mobility at pH 3.2. Both THP1 and THP2 are competed out of the antigen-antibody complex by purified thionin. The conversion of THP2 into thionin, which has been demonstrated in a pulse-chase experiment *in vivo*, is a post-translational process. As it has not been possible to detect THP1 *in vivo* it is assumed that it is converted co-translationally into THP2. Final deposition of thionin as an extrinsic membrane protein, possibly associated with the endoplasmic reticulum, has been tentatively established on the basis of subcellular fractionation experiments.

Key words: barley/endosperm/*Hordeum vulgare* L./protein biosynthesis/thionin

Introduction

A single tissue, the cereal endosperm, represents >70% of the edible plant agricultural product. The study of protein synthesis, processing and deposition in this tissue is of interest in connection with the possible application of molecular techniques to its genetic manipulation for practical purposes. The endosperms of maize (Burr *et al.*, 1978; Larkins and Hurkman, 1978; Wienand and Feix, 1978; Viotti *et al.*, 1979) and barley (Brandt and Ingversen, 1978; Matthews and Miflin, 1980; Jonassen *et al.*, 1981; Paz-Ares *et al.*, 1983) have been the most studied in this context. Similar studies are in progress with wheat (Greene, 1981; Okita and Greene, 1982; Donovan *et al.*, 1982), rice (Yamagata *et al.*, 1982a) and oats (Luthe and Peterson, 1977; Mathashewski *et al.*, 1982).

Although most of the initial attention has been focused on the storage proteins, which are of poor nutritional quality, more recently interest has been excited by other abundant endosperm proteins with higher proportions of essential amino acids and probably more amenable to genetic manipulations than the multigene-encoded reserve proteins. Thionins are polypeptides of mol. wt. ~5000, with high lysine and cystine contents, which represent up to 4% of the endosperm protein in many Gramineae (Balls *et al.*, 1942; Carbonero and García-Olmedo, 1969; Redman and Fisher, 1969; Hernández-Lucas *et al.*, 1978). Several of them have been sequenced (Othani *et al.*, 1975, 1977; Mak and Jones, 1976; Jones and Mak, 1977). Genetic and biochemical studies have shown that in hexaploid wheat (*Triticum aestivum* L.) and in diploid rye (*Secale cereale*, L) there is only one thionin variant per genome (Fernández de Caleya *et al.*, 1976; Sánchez-Monge *et al.*, 1979). However, in the case of diploid barley (*Hordeum vulgare* L.), although a single electrophoretic variant has been observed, sequence studies seem to indicate heterogeneity (see García-Olmedo *et al.*, 1982).

This report deals with the biosynthesis of thionin in barley endosperm. We present evidence that barley thionin is synthesized by membrane-bound polysomes as a much larger precursor that, after processing, yields the mature protein, which possibly remains associated with the endoplasmic reticulum (ER).

Results

Thionin synthesis during endosperm development

Thionin was analysed in samples of developing endosperm collected at different intervals after anthesis (Figure 1). Thionin accumulation, as judged from the stained electrophoretic band, was first detected at 15 days after anthesis and was practically completed at ~30 days (Figure 1A). Labelling of excised ears with [^{35}S]SO_4^{2-} indicated that as early as 8 days after anthesis there was active thionin synthesis (Figure 1B). This is in contrast with the deposition of the main storage proteins, the B- and C-hordeins, which starts at a later stage and proceeds throughout endosperm development (Paz-Ares *et al.*, 1983; and Figure 1A).

To identify possible thionin precursor(s), ears collected at 20 days after anthesis were pulse-labelled with [^{35}S]SO_4^{2-} and the products that reacted with monospecific antibodies (A-TH) raised against the mature protein were selected, alkylated, and fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) (Figure 2). Total and trichloroacetic acid insoluble radioactivity accumulated in endosperm reached a plateau at 48 h (Figure 2A). After a 3 h chase, only one product (THP2, see Discussion) with an apparent mol. wt. for its alkylated derivative of 17 400 was detected. The label in this product increased at 24 h and diminished by 48 h, while the mature protein became increasingly labelled (Figure 2B).

In vitro translation

Translation of poly(A)$^+$ RNAs purified from free and membrane-bound polysomes from 20 day endosperm was carried out in the wheat-germ cell-free system, using [^{35}S]-cysteine as a label. The wheat germ cell-free system is practically a homologous system since thionin is also synthesized in wheat. The translation products immunoprecipitated with A-TH were fractionated by PAGE at pH 3.2 in the presence of 2-mercaptoethanol (Figure 3). Only the poly(A)$^+$ RNA from membrane-bound polysomes yielded a cross-reacting product (THP1, see Discussion), but it had a much lower electrophoretic mobility than the mature protein. Poly(A)$^+$ RNA from membrane-bound polysomes was fractionated by

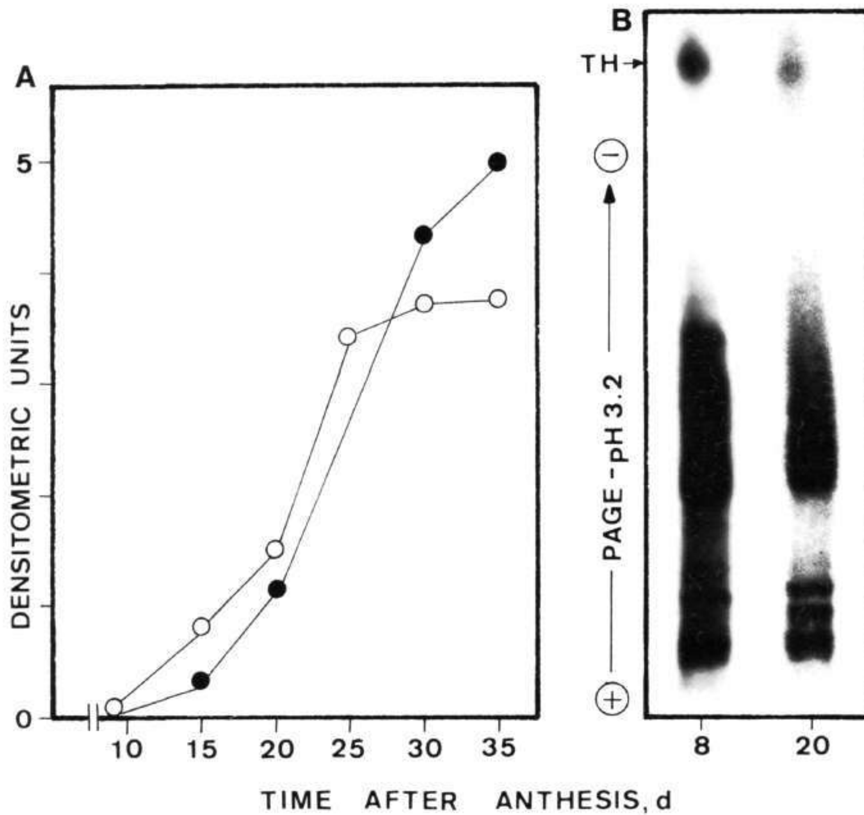

Fig. 1. Thionin synthesis during endosperm development. **(A)** Relative amounts of thionin (—○—○—) were quantitated by densitometry of the stained band after PAGE-pH 3.2 in endosperm samples collected at different times after anthesis. Hordeins (—●—●—) were similarly quantitated after SDS-PAGE in the same samples. Different arbitrary scales have been used in each case. **(B)** PAGE-pH 3.2 and fluorography of endosperm extracts. Ears were collected at 8 and 20 days after anthesis, labelled for 48 h with $[^{35}S]SO_4^{2-}$ and freeze-dried. Endosperms were separated by hand-dissection. Purified thionin (TH) was run in parallel and, after staining, its position in the gel was marked with radioactive ink.

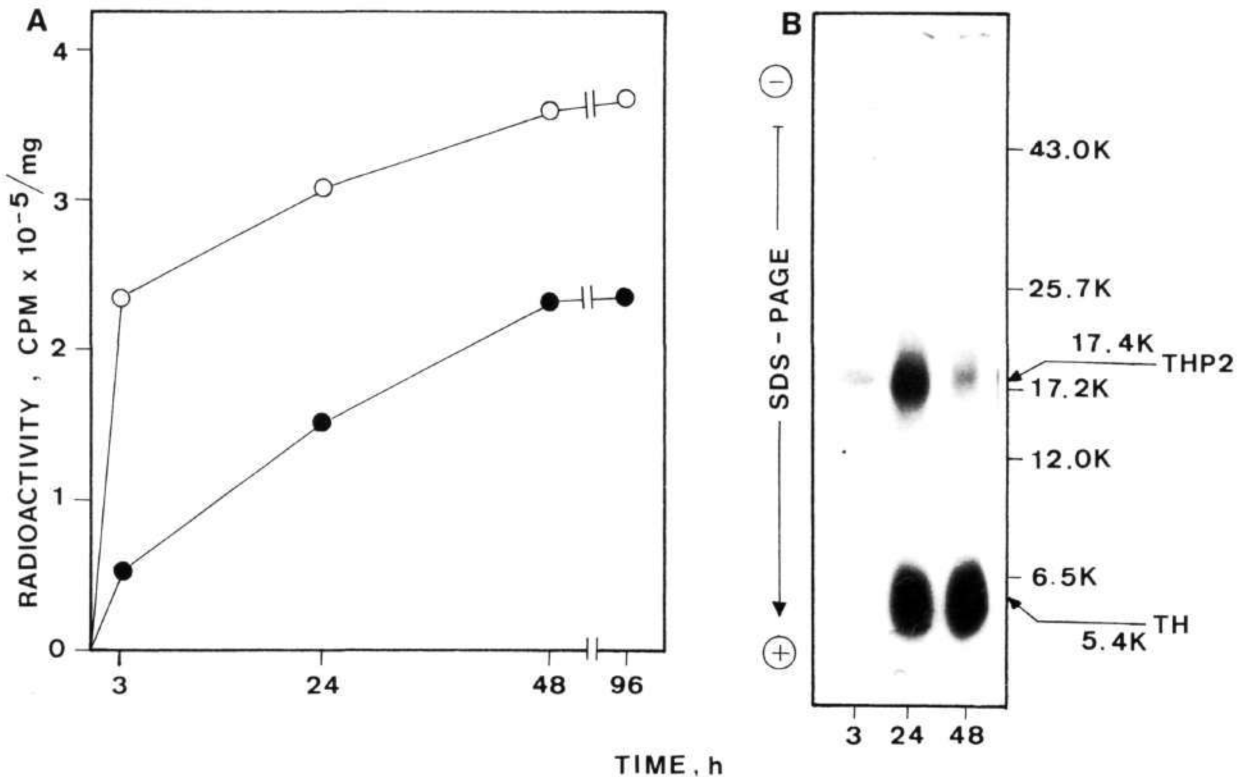

Fig. 2. Pulse-chase experiment. Time-course of $[^{35}S]SO_4^{2-}$ incorporation into proteins of 20 day barley endosperm. Samples were collected at 3, 24, 48 and 96 h after label was added. **(A)** Total (—○—○—) and trichloroacetic acid insoluble c.p.m./mg (—●—●—) incorporated into endosperm at different times. **(B)** Proteins immunoprecipitated with monospecific antibodies (A-TH) at the successive stages were alkylated and subjected to SDS-PAGE and fluorography. Purified thionin (TH) was also alkylated and run in parallel. Its position and those of the protein standards used were marked in the gel with radioactive ink. The apparent mol. wt. of the thionin precursor (THP2) in its alkylated form is 17 400 (K = x1000).

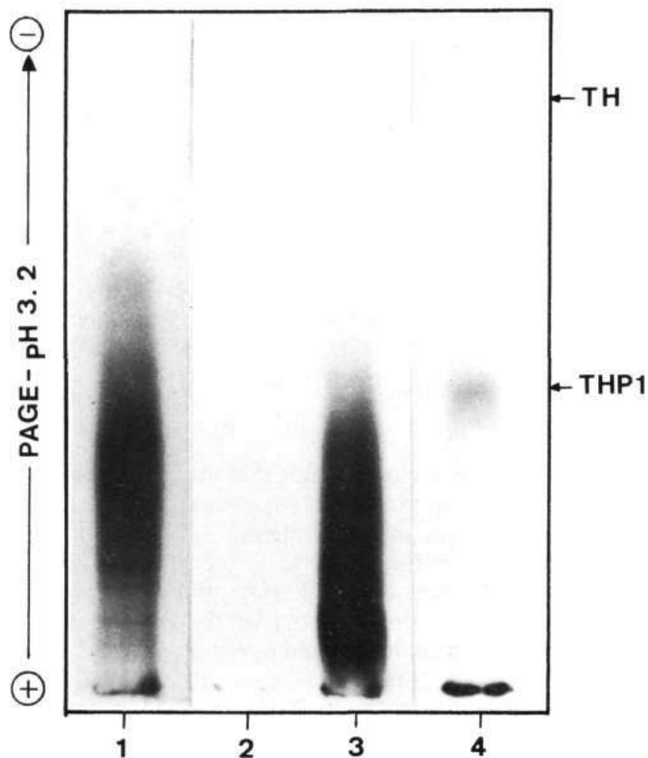

Fig. 3. PAGE-pH 3.2 and fluorography of translation products directed by poly(A)$^+$ RNAs purified from free and membrane-bound polysomes from 20 day endosperm in a wheat germ cell-free system. **(1)** Total free poly(A)$^+$ RNA translation products. **(2)** Immunoprecipitate from previous sample. **(3)** Total bound poly(A)$^+$ RNA translation products. **(4)** Immunoprecipitate from previous sample. Purified thionin (TH) was run in parallel and its position marked with radioactive ink.

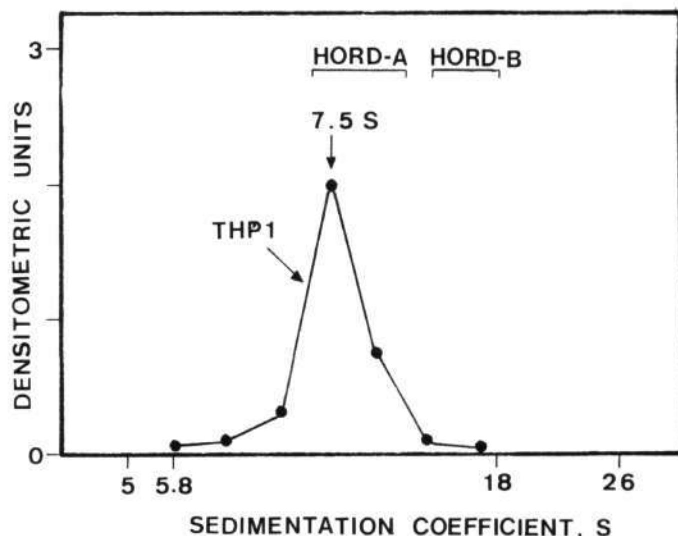

Fig. 4. Sucrose-gradient (10 – 35%, w/v) centrifugation of poly(A)$^+$ RNA (150 μg) from membrane-bound polysomes. Centrifugation was carried out in 10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.5% SDS at 100 000 g_{av} for 26 h (18°C) in a Beckman SW-27 rotor and 18 fractions of 2 ml each were collected. An aliquot of 4% from each fraction was translated and products reacting with monospecific antibodies were precipitated, separated by PAGE-pH 3.2 and fluorographed. The positions of mRNAs for A- and B-hordeins, in the gradient were determined as in Paz-Ares *et al.* (1983). Messenger activity for THP1 was evaluated in the fluorograph by densitometry.

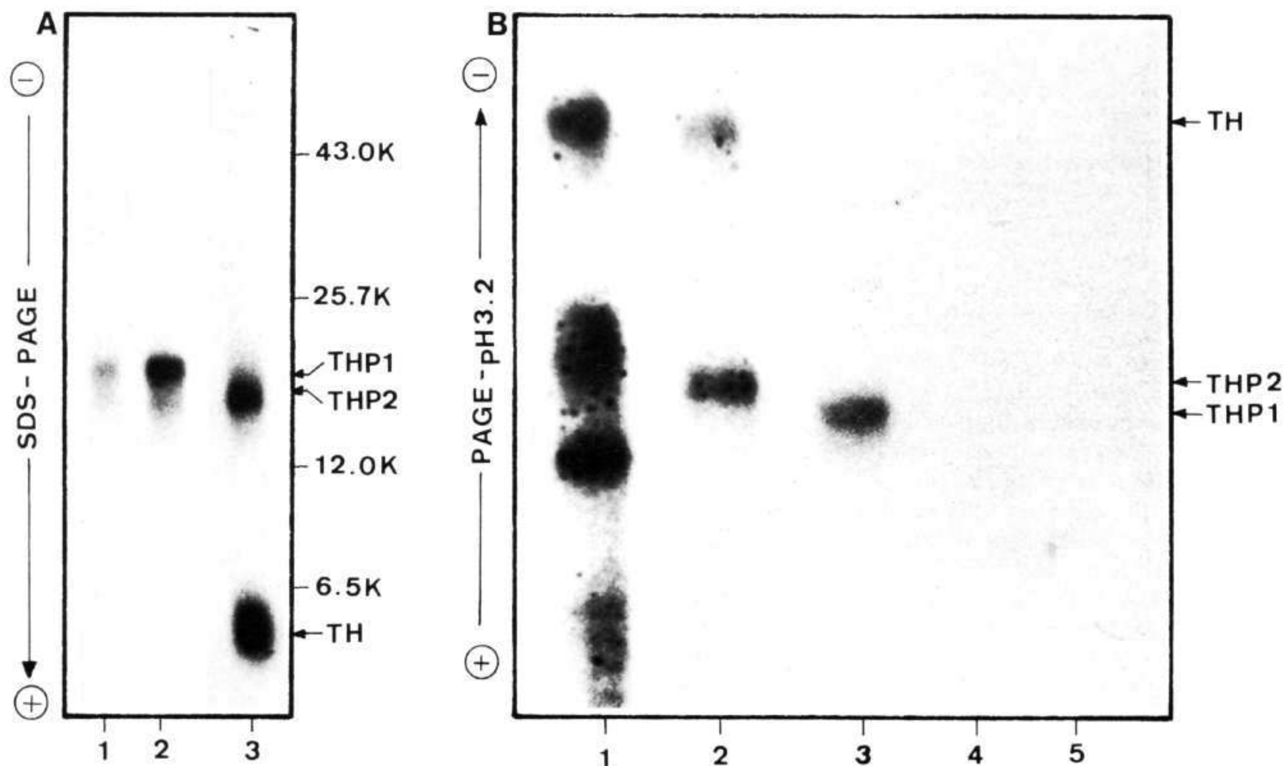

Fig. 5. Comparison of *in vivo* and *in vitro* products selected with monospecific antibodies and displacement of these products from the antigen-antibody complex by purified thionin. **(A)** SDS-PAGE of alkylated products: **(1)** *in vitro* precursor THP1 labelled with [^{35}S]methionine; **(2)** *idem* with [^{35}S]cysteine; **(3)** *in vivo* products, THP2 and TH. **(B)** PAGE-pH 3.2 of reduced, non-alkylated products: **(1)** total *in vivo* extracts; **(2)** *in vivo* products, THP2 and TH; **(3)** *in vitro* precursor THP1; **(4)** as in **lane 2** plus 5 μg of unlabelled thionin; **(5)** as in **lane 3** plus 5 μg of unlabelled thionin. Other details as in Figures 2 and 3.

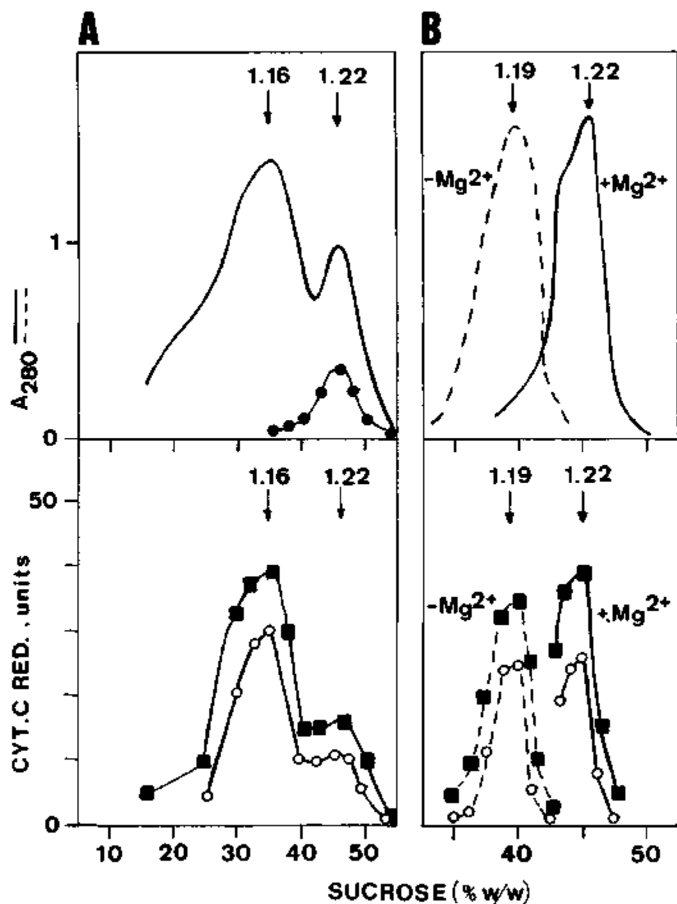

Fig. 6. Subcellular location of thionin. **(A)** Co-sedimentation of cytochrome c reductase and thionin. The membrane fraction from a 20 day endosperm homogenate was sedimented to equilibrium in a 10–50% (w/w) sucrose gradient (50 mM Tricine, pH 7.5, 100 mM K acetate, 10 mM Mg acetate). **(B)** Fractions between 40% and 50% sucrose from three gradients were pooled and centrifuged again in the same conditions ($+Mg^{2+}$) or in the presence of 10 mM EDTA ($-Mg^{2+}$). Thionin (—○—○—) and hordeins (—●—●—) were quantitated as indicated in Figure 1; arbitrary units are not represented in the axis. Cytochrome c reductase (—■—■—) activity is expressed in nmol/ml/min.

ultracentrifugation in a 10–35% sucrose gradient and most of the mRNA activity for THP1 was found in a fraction corresponding to a sedimentation coefficient of 7.5S (Figure 4).

Comparisons of in vivo and in vitro products

In vivo and *in vitro* products were compared by SDS-PAGE as their alkylated derivatives and by PAGE-pH 3.2 in the presence of 2% 2-mercaptoethanol (Figure 5). The *in vitro* product, THP1, was labelled not only by [^{35}S]cysteine but also by [^{35}S]methionine (an amino acid that is not present in the mature protein) and it has an apparent mol. wt. which is higher by ~400 than that of THP2 (Figure 5A). The two products, THP1 and THP2, which also have different electrophoretic mobility in PAGE-pH 3.2, were displaced from the antibody complex by an excess of unlabelled, purified thionin (Figure 5B).

Subcellular location

The 500 g_{av} supernatant from a homogenate of 20 day endosperms was centrifuged over a double sucrose cushion (20%/50%; w/w) at 36 000 g_{av} for 1 h and thionin was quantitatively recovered at the 50% interface. This fraction was subsequently centrifuged on a 10–50% (w/w) sucrose gradient (Figure 6A) and two absorbance peaks were obtained at densities 1.22 and 1.16 g/cm^3. The distribution of thionin in the gradient coincided with that of a specific ER marker, cytochrome c reductase (NADP-dependent; antimycin A-resistant). Fractions from the gradient between 40% and 50% sucrose were pooled and ultracentrifuged again under the same conditions ($+Mg^{2+}$) and in the presence of EDTA ($-Mg^{2+}$). Both thionin and the ER marker banded at a lower density in the absence of Mg^{2+} (Figure 6B).

Brief treatments (20 min, 4°C) of the particulate fraction with 0.5 M NaCl or 1% Nonidet-P40 (NP-40) did not affect protein bodies (checked by electron microscopy; not shown) but quantitatively extracted thionin.

Discussion

The present experiments indicate that thionin is synthesized by membrane-bound polysomes as a larger precursor that is processed to yield the mature protein.

Only one polypeptide, THP1, is recognized by monospecific antibodies among the *in vitro* translation products. This polypeptide is competed out of the antigen-antibody complex by unlabelled thionin and does not seem to be an aggregated form of the mature protein, as judged from the electrophoretic conditions used. It should also be pointed out that the apparent size of the THP1 polypeptide is compatible with that of its corresponding mRNA.

In the *in vivo* pulse-chase labelling experiments, a similar polypeptide, THP2, is also selected by monospecific antibodies and competed out by purified thionin. The two polypeptides, THP1 and THP2, slightly differ in their apparent mol. wt. and in the electrophoretic mobility in PAGE-pH 3.2 (2% mercaptoethanol) of their reduced, non-alkylated forms. The structural basis of the observed differences could involve a few amino acids, possibly as little as the N-terminal methionine, or some other type of modification. The possibility of THP1 being an artefact of the *in vitro* translation system seems to be unlikely, although it cannot be excluded.

The immunological evidence suggests that both THP1 and THP2 are thionin precursors. In the case of THP2, this hypothesis is further supported by the results of the pulse-chase experiment, which show that the conversion of THP2 into mature protein is a post-translational process that involves a considerable reduction in size, from ~17 000 to 5000. Our failure to detect THP1 *in vivo*, under conditions in which most of the labelled amino acid present in the endosperm is not yet incorporated into proteins, suggests that THP1 is converted into THP2 co-translationally. However, we have not yet been able to fully confirm this in an appropriate *in vitro* system.

No detailed study of the subcellular location of thionin has been carried out, but the observations reported here clearly indicate that it is associated with the particulate fraction and that it is not located in the protein bodies because most of it does not co-sediment with the hordeins and because it is possible to obtain protein bodies without thionin by a brief treatment with the non-ionic detergent NP-40. Our results further suggest that thionin is extrinsically associated with the endoplasmic reticulum: (i) its distribution in density gradients is coincident with that of an ER-specific marker (cytochrome c reductase, NADPH, antimycin A-resistant; see Tolbert, 1974), both in the presence and in the absence of Mg^{2+}; (ii) it is solubilized both by 0.5 M NaCl, a treatment that does not

disrupt the ER, and by 1% NP-40, which does solubilize the ER.

The pathway of protein synthesis, processing and deposition, exemplified by thionin, differs from those previously described for other barley endosperm proteins: i.e., (i) the synthesis of many cytosol albumins and globulins by free polysomes (Brandt and Ingversen, 1976); (ii) the synthesis of storage B- and C-hordeins by membrane-bound polysomes; with co-translational excision of signal peptides, and deposition in the protein bodies (Cameron-Mills *et al.*, 1978; Brandt and Ingversen, 1978; Matthews and Miflin, 1980); (iii) the synthesis of A-hordeins, with co-translational processing, and export into the cytosol (Paz-Ares *et al.*, 1983). Although the synthesis by membrane-bound polysomes of thionin and of A-, B- and C-hordeins is initiated and terminated at different moments during endosperm development, this occurs simultaneously during the period between ~15 days and 30 days after anthesis. This implies that a variety of appropriate signals must exist in the different proteins to direct them through their different processing and deposition pathways.

Post-translational processing with drastic reduction in size has been recently described for various proteins in cotton (Dure and Galau, 1981), soybean (Sengupta *et al.*, 1981), pea (Chrispeels *et al.*, 1982), rice (Yamagata *et al.*, 1982a, 1982b) and oats (Mathashewski *et al.*, 1982). However, little is known about the processing mechanisms or even if the same type of processing occurs in all the cases described. If, in fact, the processing is similar, some long term objectives, such as the expression of alien globulin genes in wheat or barley, would be considerably facilitated.

Materials and methods

Plant material

Endosperms from *H. vulgare* L., cv. Bomi, were obtained from ears collected at different times after anthesis by hand dissection or by mechanical extrusion into liquid nitrogen (O'Dell and Thompson, 1982).

In vivo protein synthesis

Proteins synthesized by developing endosperm were labelled with $[^{35}S]SO_4^{2-}$ (1100 Ci/nmol, New England Nuclear) essentially as described by Donovan and Lee (1977): ears were cut at different stages of development (5–10 cm of stem) and placed in conical tubes with 0.5 ml of a sulphur-free nutrient solution containing 100–300 μCi of the radioactive precursor. After the label had been absorbed (~30 min), two successive 0.5 ml aliquots of the same nutrient solution without label were added and then the ear was transferred to nutrient solution at 4°C.

Purification of mRNA

Total RNA from free and from membrane-bound polysomes was prepared according to Larkins *et al.* (1976), with minor modifications (Paz-Ares *et al.*, 1983). The RNA was enriched in the poly(A)$^+$-containing fraction by one or two passages through a column of oligo(dT)-cellulose (PL-Biochemical, type 7) according to Aviv and Leder (1972), as specified by Paz-Ares *et al.* (1983).

Poly(A)$^+$ RNA from initially membrane-bound polysomes was fractionated in a 10–35% (w/v) linear sucrose gradient in 10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.5% SDS. The sample, 150 μg of RNA in 0.5 ml of the same buffer, without sucrose, was heated 4 min at 80°C, rapidly cooled on ice and loaded in the gradient. Centrifugation was carried out in a SW-27 rotor at 100 000 g_{av} for 26 h at 18°C. Fractions (2 ml each) were collected and precipitated with 1/10 volume of 2 M Na acetate and two volumes of ethanol. RNAs of 5S, 5.8S, 18S and 26S were centrifuged in parallel for calibration purposes.

In vitro protein synthesis

In vitro translation was carried out according to Marcu and Dudock (1974), with minor modifications (Paz-Ares *et al.*, 1983). The labelled amino acid used was either $[^{35}S]$cysteine (900 Ci/mmol, New England Nuclear) or $[^{35}S]$-methionine (1200 Ci/mmol, Amersham International p.l.c.). Two different sources of equally effective wheat germ were used: commercial wheat germ from General Mills (USA) and hand-dissected wheat embryos from *T. turgidum*, cv. Ledesma. Each preparation was optimized for cation concentrations. The conditions varied slightly for each lot, but were always close to 100 mM K^+, 2 mM Mg^{2+} and 70 mM spermine.

Preparation of monospecific antibodies

Barley thionin was purified following the method previously described for wheat thionins (García-Olmedo *et al.*, 1968). The protein was coupled to bovine serum albumin and injected s.c. and i.p. to adult female rabbits, together with Freund's complete adjuvant (Difco) at 3–4 week intervals (Tai and Chey, 1978). Initial doses were of 1 mg/injection. Sera were prepared when useful antibody titers were obtained and stored at −20°C. Antibodies were purified by precipitation with 40% saturated $(NH_4)_2SO_4$ and passage through a thionin-Sepharose affinity column prepared according to Shapiro *et al.* (1974).

Immunoprecipitation of in vivo and in vitro products

The following method, based on that of Shapiro and Young (1981), was used for proteins labelled *in vivo*: 1 mg of meal from $[^{35}S]SO_4^{2-}$-labelled endosperms was extracted with a mixture of 100 μl of petroleum ether (Carlo Erba, 40°–60°C) and 300 μl of 1 N HCl in ethanol, and reextracted with petroleum ether. The delipidated meal was extracted with 30 μl of 0.5 M NaCl for 1 h, made up to 200 μl phosphate buffered saline (PBS) (0.15 M NaCl, 0.01 M sodium phosphate, pH 7.2), left in the cold (4°C) during 2 h, and centrifuged. The monospecific antibody preparation (10 μl) was added to the supernatant and the mixture incubated at 37°C for 1 h and at 4°C for 2–15 h. Finally, 80 μl of IgG Sorb (The Enzyme Center, Inc.) were added and, after a 30 min incubation at 4°C, the precipitate was centrifuged (10 000 g_{av}) through a cushion of 1 M sucrose, 1% Triton X-100 and 1% Na-deoxycholate in PBS. The precipitates were washed three times in PBS, 1% Triton X-100, 1% Na-deoxycholate and, finally, three times in PBS, before they were dissociated for electrophoresis.

The same procedure was followed for *in vitro* products, except that a precipitation with pre-immune serum preceded that carried out with monospecific antibodies and all washing buffers included 50 μM cysteine or methionine.

For the competition experiments with purified thionin, the appropriate quantity of protein was added to the mixture immediately before adding the first antibody.

Electrophoresis and fluorography

Two different electrophoretic systems were used: PAGE, at pH 3.2, in the presence of excess 2-mercaptoethanol, and SDS-PAGE. The gels for electrophoresis at acid pH (7.5% w/v total acrylamide) were polymerized in distilled water, and incubated overnight in an excess of acid buffer (0.015 M aluminium lactate/lactic acid buffer, pH 3.2, 3 M urea, 2% 2-mercaptoethanol). Antibody-treated samples were disrupted in an alkaline buffer (0.025 M Tris/glycine buffer, pH 9, 9 M urea, 10% 2-mercaptoethanol) during 1 h at 37°C. Endosperm extracts were made 10% 2-mercaptoethanol before electrophoresis. Gels (2 mm thick) were run for 3 h at 20 V/cm in the cold (4°C) and stained with Coomassie Brilliant Blue (Blakesley and Boezi, 1977). SDS-PAGE was performed following the method of Shapiro *et al.* (1967), as recommended by Bethesda Research Laboratories (1980) for low mol. wt. proteins. Separating gel was 12.5% w/v total acrylamide and stacking gel was 4%. Electrophoresis was run at room temperature until the tracking dye (Bromophenol Blue) reached the bottom and the gels were stained with Coomassie Brilliant Blue. Antibody-treated samples were disrupted in sample buffer plus 10 mM dithiothreitol for 1 h at 37°C and alkylated with 30 mM sodium iodoacetate for 1 h at 37°C. Samples were boiled for 3 min before electrophoresis.

Fluorography was performed using 2,5-diphenyloxazol as scintillator (Bonner and Laskey, 1974; Laskey and Mills, 1975). The dried gels were exposed at −70°C, for different periods of time.

Fractionation of developing endosperm

Endosperms were homogenized in 50 mM Tricine, pH 7.5, 100 mM K acetate, 10 mM Mg acetate (Miflin *et al.*, 1981) and the homogenate centrifuged at 500 g_{av} for 5 min. The supernatant was centrifuged over a 30%/50% sucrose (w/w) discontinuous gradient in the same buffer at 36 000 g_{av} for 1 h in a SW-27 rotor. The fraction collected at the 30%/50% interphase was further fractionated in a 10–50% sucrose (w/w) continuous gradient at 75 000 g_{av} for 1 h in a SW-41 rotor. Aliquots from each fraction were assayed for cytochrome c reductase (NADP-dependent, antimycin A-resistant) as described (Miflin *et al.*, 1981). The remainder of each fraction was diluted with buffer, pelleted at 20 000 g_{av} for 30 min, and freeze-dried. Thionin and hordeins were quantitated by densitometry of electrophoretic bands according to Carbonero *et al.* (1980).

Acknowledgements

This research was supported by grants from the 'Fundación Ramón Areces' and from the 'Comisión Asesora de Investigación Científica y Técnica'.

References

Aviv,H. and Leder,P. (1972) *Proc. Natl. Acad. Sci. USA*, **69**, 1408-1412.
Balls,A.K., Hale,W.S. and Harris,T.H. (1942) *Cereal Chem.*, **19**, 279-288.
Bethesda Research Laboratories (1980) *Focus*, **2**, 6.
Blakesley,R.W. and Boezi,J.A. (1977) *Anal. Biochem.*, **82**, 580-582.
Bonner,W.M. and Laskey,R.A. (1974) *Eur. J. Biochem.*, **46**, 83-88.
Brandt,A. and Ingversen,J. (1976) *Carlsberg Res. Commun.*, **41**, 311-320.
Brandt,A. and Ingversen,J. (1978) *Carlsberg Res. Commun.*, **43**, 451-469.
Burr,B., Burr,F.A., Rubenstein,I. and Simon,M.N. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 696-700.
Cameron-Mills,V., Ingversen,J. and Brandt,A. (1978) *Carlsberg Res. Commun.*, **43**, 91-102.
Carbonero,P. and García-Olmedo,F. (1969) *Experientia*, **25**, 1110.
Carbonero,P., García-Olmedo,F. and Hernández-Lucas,C. (1980) *J. Agric. Food Chem.*, **28**, 399-402.
Chrispeels,M.J., Higgins,T.J.V. and Spencer,D. (1982) *J. Cell Biol.*, **93**, 306-313.
Donovan,G.R. and Lee,J.W. (1977) *Plant Sci. Lett.*, **9**, 107-113.
Donovan,G.R., Lee,J.W. and Longhurst,T.J. (1982) *Aust. J. Plant Physiol.*, **9**, 59-68.
Dure,L.,III and Galau,G.A. (1981) *Plant Physiol.*, **68**, 187-194.
Fernández de Caleya,R., Hernández-Lucas,C., Carbonero,P. and García-Olmedo,F. (1976) *Genetics*, **83**, 687-699.
García-Olmedo,F., Sotelo,I. and García-Faure,R. (1968) *An. Inst. Invest. Agron.*, **17**, 433-443.
García-Olmedo,F., Carbonero,P. and Jones,B.L. (1982) *Adv. Cereal Sci. Technol.*, **5**, 1-47.
Greene,F.C. (1981) *Plant Physiol.*, **68**, 778-783.
Hernández-Lucas,C., Carbonero,P. and García-Olmedo,F. (1978) *J. Agric. Food Chem.*, **26**, 794-796.
Jonassen,I., Ingversen,J. and Brandt,A. (1981) *Carlsberg Res. Commun.*, **46**, 175-181.
Jones,B.L. and Mak,A.S. (1977) *Cereal Chem.*, **54**, 511-523.
Larkins,B.A. and Hurkman,W.J. (1978) *Plant Physiol.*, **62**, 256-263.
Larkins,B.A., Jones,R.A. and Tsai,C.Y. (1976) *Biochemistry (Wash.)*, **15**, 5506-5511.
Laskey,R.A. and Mills,A.D. (1975) *Eur. J. Biochem.*, **56**, 335-341.
Luthe,D.S. and Peterson,D.M. (1977) *Plant Physiol.*, **59**, 836-841.
Mak,A.S. and Jones,B.L. (1976) *Can. J. Biochem.*, **54**, 835-842.
Marcu,K. and Dudock,B. (1974) *Nucleic Acids Res.*, **1**, 1385-1397.
Mathashewski,G.J., Adeli,K., Altosaar,I., Shewry,P.R. and Miflin,B.J. (1982) *FEBS Lett.*, **145**, 208-212.
Matthews,J.A. and Miflin,B.J. (1980) *Planta*, **149**, 262-268.
Miflin,B.J., Burgess,S.R. and Shewry,P.R. (1981) *J. Exp. Bot.*, **32**, 199-219.
O'Dell,M. and Thompson,R.D. (1982) *J. Sci. Food Agric.*, **33**, 419-420.
Okita,T.W. and Greene,F.C. (1982) *Plant Physiol.*, **69**, 834-839.
Othani,S., Okada,T., Kagamiyama,H. and Yoshizumi,H. (1975) *Agric. Biol. Chem.*, **39**, 2269-2270.
Othani,S., Okada,T., Yoshizumi,H. and Kagamiyama,H. (1977) *J. Biochem. (Tokyo)*, **82**, 753-767.
Paz-Ares,J., Ponz,F., Aragoncillo,C., Hernández-Lucas,C., Salcedo,G., Carbonero,P. and García-Olmedo,F. (1983) *Planta*, **157**, 74-80.
Redman,G.D. and Fisher,N. (1969) *J. Sci. Food Agric.*, **20**, 427-432.
Sánchez-Monge,R., Delibes,A., Hernández-Lucas,C., Carbonero,P. and García-Olmedo,F. (1979) *Theor. Appl. Genet.*, **54**, 61-63.
Sengupta,C., Deluca,V., Bailey,D.S. and Verma,D.P.S. (1981) *Plant Mol. Biol.*, **1**, 19-34.
Shapiro,A.L., Viñuela,E. and Maizel,J.B. (1967) *Biochem. Biophys. Res. Commun.*, **28**, 815-820.
Shapiro,D.J., Taylor,J.M., McKnight,G.S., Palacios,R., González,C., Kiely,M.L. and Schimke,R.T. (1974) *J. Biol. Chem.*, **249**, 3665-3671.
Shapiro,S.Z. and Young,J.R. (1981) *J. Biol. Chem.*, **256**, 1495-1498.
Tai,H.H. and Chey,W.Y. (1978) *Anal. Biochem.*, **87**, 350-358.
Tolbert,N.E. (1974) *Methods Enzymol.*, **31**, 734-746.
Viotti,A., Sala,E., Marotta,R., Alberi,P., Balducci,C. and Soave,C. (1979) *Eur. J. Biochem.*, **102**, 211-222.
Wienand,U. and Feix,G. (1978) *Eur. J. Biochem.*, **92**, 605-611.
Yamagata,H., Sugimoto,T., Tanaka,K. and Kasai,Z. (1982a) *Plant Physiol.*, **70**, 1094-1100.
Yamagata,H., Tanaka,K. and Kasai,Z. (1982b) *Agric. Biol. Chem.*, **46**, 321-322.