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Selective proteolysis of the wheat Em polypeptide

Identification of an endopeptidase activity in germinating wheat embryos

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The 'Em' polypeptide is the most abundant cytosolic polypeptide in mature wheat embryos. It is selectively and completely degraded within the first 24 h of germination. Extracts from germinated embryos contain endopeptidase activities which degrade the Em polypeptide These are separable into a major and minor component by ion-exchange chromatography and the use of inhibitors shows the major component to be a cysteine proteinase. This activity shows a strong preference for the Em polypeptide as a substrate, being inactive against polypeptides which are not developmentally regulated and showing only low activity towards developmentally related, but otherwise nonhomologous 'dehydrin' polypeptides.

Em polypeptide; Cysteine proteinase; Wheat embryo; Germination

1. INTRODUCTION

The most abundant cytosolic wheat embryo protein is the 'Em' (early methionine-labelled) polypeptide found in mature (dry) embryos [1]. Em synthesis is initiated during embryogenesis, in response to the plant growth regulator abscisic acid (ABA) [2,3]. Whilst it may fulfil a storage function, its physical properties [4] and the induction of Em expression by the imposition of osmotic stress [5] suggest that it is one of a small number of 'anhydrobiosis'-related gene products whose function is to confer desiccation-tolerance on the embryo, which undergoes an obligatory period of dehydration in the course of its development [4,6]. Levels of the Em polypeptide increase rapidly in the cytosol and persist throughout desiccation of the grain. Upon subsequent imbibition, both the polypeptide and its mRNA are rapidly degraded, completely disappearing during the 24 h following the first contact of the embryo with water [1,7]. The degradation of the Em polypeptide appears selective, in that other cytosolic species, which are not developmentally regulated, remain stable.

Because of the striking pattern of expression of the Em genes, we have examined the apparent selectivity of Em degradation during germination. In this report, we identify a proteolytic activity present in imbibing wheat embryos which appears preferentially to degrade the Em polypeptide, whilst exhibiting no activity towards a number of other cytosolic proteins.

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Abbreviations: ABA: abscisic acid; Em: early methionine-labelled protein; kDa: kilodaltons; M_r: relative molecular mass; PGK: phosphoglycerate kinase; *p*-HMB: *p*-hydroxymercuribenzoic acid; PMSF: phenylmethysulphonyl fluoride; TLCK: tosyl-L-lysine-chloromethylketone.

2. MATERIALS AND METHODS

2.1. *Plant material*

Embryos were dissected from grains of *Triticum aestivum* L. var. Axona (Kenneth Wilson (Grain) Ltd., Leeds, UK) which had been surface-sterilised with sodium hypochlorite (1% free Cl^2) for 10 min, washed and imbibed on filter papers soaked with water (25°C).

2.2. *Preparation of extracts*

Embryos were homogenised in 10 mM Tris-Cl/1 mM EDTA, pH 7.0 (2 ml/g embryos; 0°C) and the homogenate clarified by centrifugation (12,000 × *g*, 10 min, 0°C). The clarified homogenates were used immediately or stored frozen at −20°C prior to further fractionation by chromatography on DEAE-cellulose (7 cm column: 9 ml bed volume eluted with a continuous gradient of NaCl (0–2 M) in 10 mM Tris-Cl/1 mM EDTA, pH 7.0). Fractions were assayed for their ability to degrade ^{35}S-labelled Em polypeptide.

2.3. *Proteolysis assays*

Extracts were tested for their ability to degrade radiochemically pure ^{35}S-labelled polypeptides. Full-length cDNA sequences encoding a range of products were cloned in the plasmid pBluescript KS$^+$, and used as the templates for transcription by T3 and T7 RNA polymerases (Gibco BRL, UK), in the presence of the cap dinucleotide ^{m7}G(5′)ppp(5′)G, using the protocols recommended by the manufacturer. The resulting transcripts were used to programme an homologous, wheat-germ cell-free protein synthesising extract [8] containing [^{35}S]methionine. For assays of Em proteolysis, the reaction mixtures were heated for 5 min at 90°C to obviate contamination of the subsequent assay with traces of proteolytic activity inherent in the wheat-germ extracts; a property of the Em polypeptide is its extreme heat-stability [9]. This was also carried out when other substrates known to exhibit heat-stability ('dehydrins') were tested. Aliquots of the translation products were used as the substrate in proteolysis assays.

Assays comprised 20 μl 'substrate mix' (3–5 × 10^3 cpm translation product in 25 mM citrate buffer pH 6/2.5 mM $MgCl_2$) and 30 μl of embryonic extract. Incubation was at 25°C for the times indicated in the text. Following incubation, the reactions were stopped by addition of 50 μl SDS sample buffer (12% v/v glycerol/4% w/v SDS/2% v/v β-mercaptoethanol) and heated at 95°C for 2 min. Reaction products were analysed by electrophoresis in dodecylsulphate polyacrylamide gels designed for the optimal resolution of small peptides [10]. Labelled species were identified by fluorography of dried gels, following impregnation of the gels with Amplify (Amersham International plc, UK). Quantitative estimates of Em and its digestion products were obtained by densitometry of the fluorograms, using an LKB Ultroscan densitometer.

3. RESULTS AND DISCUSSION

3.1. *Germinating embryos contain Em-endopeptidase activity*

The Em polypeptide is readily identifiable among dodecylsulphate polyacrylamide gel separations of soluble wheat embryo proteins. The disappearance of this polypeptide during the course of imbibition of dry seeds is clearly evident in Fig. 1. In the seed lot used in this study, the endogenous Em polypeptide underwent almost complete degradation, in vivo, within 24 h of water uptake. In choosing conditions for the extraction of proteinase activities with potentially Em-directed activity, we therefore selected an incubation time (16 h) which allowed approximately half-maximal Em degradation to occur, reasoning that such a stage would represent a peak in endogenous proteinase activity. Because germinating wheat embryos have been shown to contain high, and increasing, levels of proteolytic activity when tested with a variety of heterologous substrates [10–12], we deliberately set out to devise an assay which would enable an Em-specific activity to be recognised. This assay was based on the synthesis, in vitro, of a radiochemically pure ^{35}S-labelled Em polypeptide, by sequential transcription and translation of an Em cDNA sequence for use as a substrate. When unfractionated, clarified embryonic homogenates were incubated with radiochemically pure Em protein, and the incubation products were analysed by dodecylsulphate gel electrophoresis and fluorography, a distinctive pattern of Em digestion products was generated (Fig. 2). Characteristically, this comprised 5 methionine-labelled peptides.

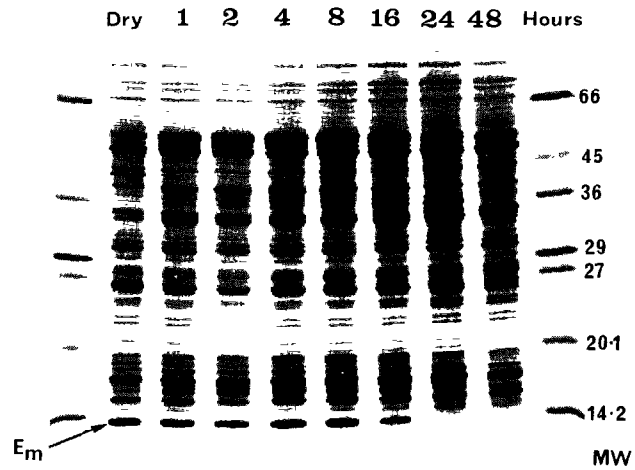

Fig. 1. Em degradation, in vivo. Total soluble polypeptides were isolated from embryos dissected from imbibing wheat grains, resolved by dodecylsulphate polyacrylamide gel electrophoresis and detected by staining with Coomassie blue. The position of the Em polypeptide is indicated. Each track is labelled according to the developmental stage of the grain (hours imbibed). The migration of molecular mass markers (MW· kDa × 10) is shown in the outermost tracks.

Fractionation of the clarified homogenate by DEAE-cellulose chromatography resolved this activity into two discrete fractions. Two peaks of proteolytic activity were recovered from the column. The first, eluting with 37.5 mM NaCl comprised only a minor proportion of the total proteolytic activity. This activity ('Fraction 1') yielded only a single cleavage product of M_r ca. 1 kDa less than that of the native Em polypeptide. The second

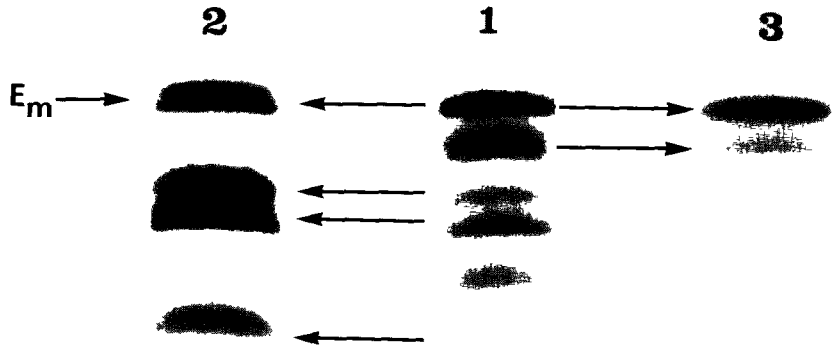

Fig. 2. Fractionation of proteolytic activities. Radiolabelled Em polypeptide was incubated with fractions eluting from a DEAE-cellulose column loaded with homogenate from 16-h imbibed embryos. The products of digestion were electrophoretically separated and detected by fluorography. Two distinct peaks of activity were identified. (1) Unfractionated activity; (2) 'Fraction 2' activity (0.5 M NaCl), (3) 'Fraction 1' activity (37.5 mM NaCl)

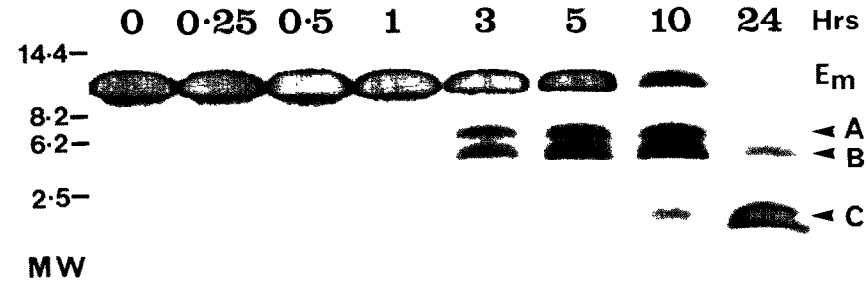

Fig. 3. Time-course of Em degradation, in vitro. Radiolabelled Em polypeptide was incubated with 'Fraction 2' proteinase activity derived from 16-h imbibed embryos, prior to electrophoretic separation and detection of the incubation products by fluorography. Each track is marked with the incubation time, in hours. The positions of the Em polypeptide, and of its derivative peptides ('A', 'B' and 'C') are indicated, as are the positions of molecular mass markers (MW: kDa × 10).

peak of activity, eluting with 0.5 M NaCl comprised the bulk of the proteolytic activity. This 'Fraction 2' activity generated peptides denoted 'A', 'B' and 'C' in Fig. 3.

Prolonged incubation of the radiolabelled Em with the 'Fraction 2' activity showed that the relative abundance of these products accumulated in a time-dependent manner, with no sign of attendant non-specific proteolysis (Fig. 3). After 3 h incubation, at least two labelled fragments of M_r ca. 6,500 ('A') and M_r ca. 5,000 ('B') were evident, and their relative abundance increased as digestion continued. The later appearance of the third peptide of M_r ca. 2,000 ('C'), clearly apparent after 10 h of incubation, and the subsequent coincident decline in the relative abundance of peptides 'A' and 'B' at longer times of digestion suggest that the smallest peptide derives from the further cleavage of the two larger components.

3.2. *The principal Em-endopeptidase is a thiol proteinase*

The nature of the Em-proteolytic activity was investigated using generic inhibitors of proteolysis. Reaction mixtures containing the 'Fraction 2' activity were supplemented with PMSF as an inhibitor of serine proteinases, TLCK as an inhibitor of both serine and cysteine proteinases, EDTA and 1,10-phenanthroline as inhibitors of metalloproteinases and *p*-HMB, iodoacetate and Cu^{2+} as inhibitors of cysteine proteinases. Following incubation and electrophoretic resolution of the digestion products the effects of the various inhibitors were quantitatively determined by estimating the extent of Em-proteolysis by scanning densitometry of the fluorograms.

The results, summarised in Table I, revealed that inhibitors of serine proteinases and metalloproteases were ineffective, whereas the sulphydryl-active reagents strongly inhibited Em degradation. This observation differentiates the Em-directed activity from the bulk of the acidic proteinase activity reported by Morris et al. [12] to be present within wheat embryos, which was largely inhibited by metal chelators: our analysis indicates that the 'Fraction 2' Em endopeptidase is a cysteine proteinase. This view was supported by the addition of dithiothreitol to the digestion mixtures. Addition of 2 mM DTT (i) stimulated the degradation of the Em protein by 100%, and (ii) was effective in ameliorating the inhibition of proteolysis by the sulphydryl-acting inhibitor *p*-HMB.

3.3. *Em-endopeptidase is not active against other cereal proteins*

The disappearance of the Em polypeptide from the cytosolic protein pool appears to be highly selective (Fig. 1). This suggests that the proteolytic mechanism responsible for its disappearance must itself be highly discriminating in its substrate specificity. If the endopeptidase activity we have identified is the principal activity responsible for Em degradation, then we would predict that it should be inactive against a range of other wheat cytosolic polypeptides not normally degraded during early germination. We tested this prediction by investigating the activity of the 'Fraction 2' proteinase towards a number of different, radiochemically pure, substrates.

Table I

Inhibition of Em-proteolysis by specific reagents

Inhibitor	Concentration (mM)	% Em degradation
PMSF	0.4	88.6
TLCK	1.0	31.9
	10.0	0
EDTA	1.0	91.7
1,10-Phenanthroline	1.0	100.5
DTT	2.0	200.0
p-HMB	1.0	17.3
p-HMB + DTT	1.0 + 2.0	47.0
Iodoacetate	1.0	27.5
	10.0	3.5
$CuCl_2$	1.8	52.7
	18.0	19.7
$CaCl_2$	18.0	95.2

Em degradation was estimated by scanning densitometry to determine the intensity of the fluorographic signal in the native Em band relative to that in the resultant peptides. The degree of proteolysis in digests containing the inhibitor was compared with that recorded in control (uninhibited) digests (100%) to obtain a percentage of Em digestion.

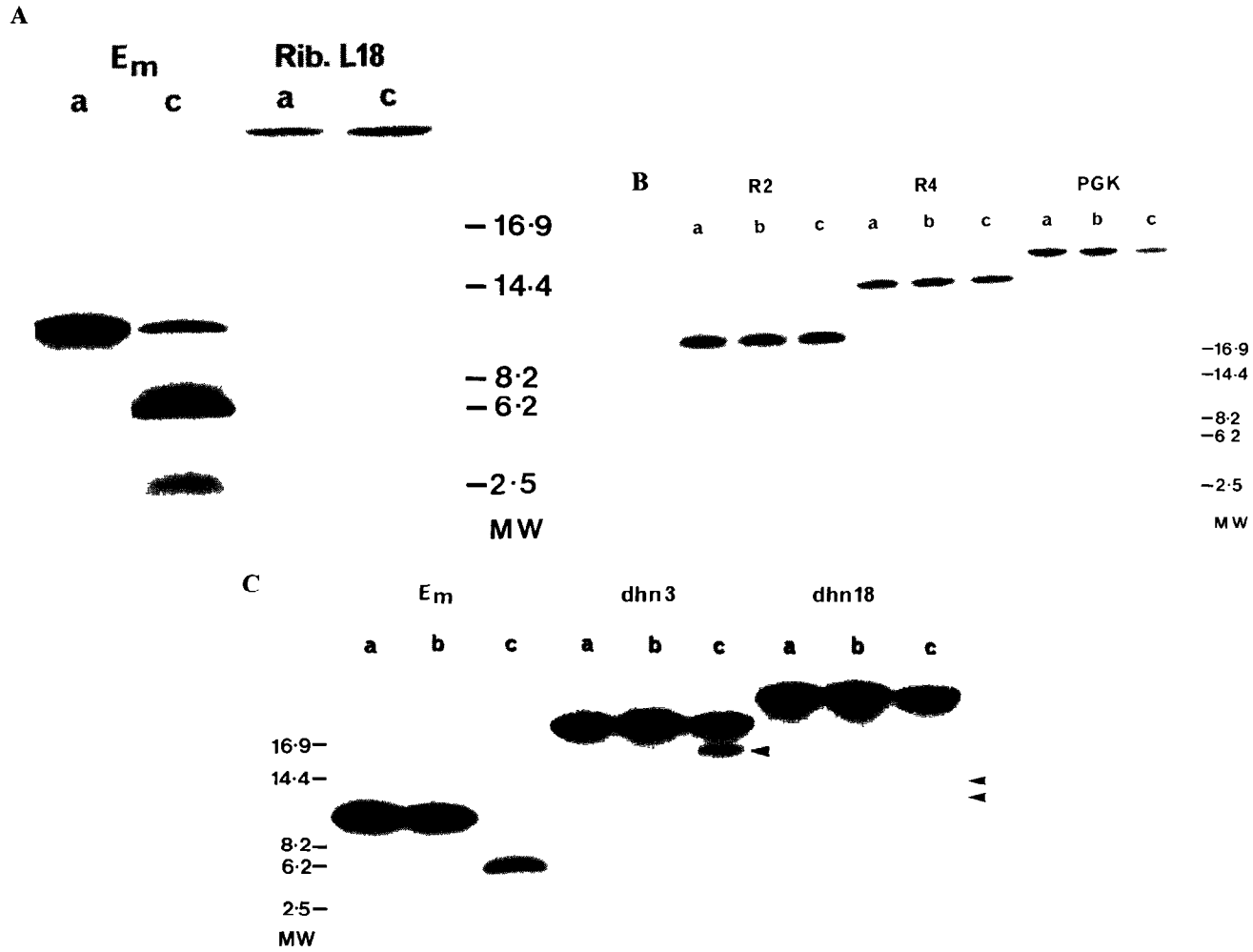

Fig. 4. Specificity of Em protease. Radiochemically pure translation products were incubated with 'Fraction 2' proteinase extract for 0 and 6 h, prior to electrophoretic separation and detection by fluorography. Groups of tracks are identified by the gene product tested: Panel A: Em = Em polypeptide; Rib. L18 = wheat ribosomal protein L18. Panel B: R2 = product of random constitutive cDNA clone pWGR2; R4 = product of random constitutive clone pWGR4; PGK = wheat cytosolic phosphoglycerate kinase. Panel C: dhn3 = maize dehydrin; dhn18 = barley dehydrin. The migration of molecular mass markers is indicated (MW: kDa × 10). Tracks marked 'a' represent undigested controls. Tracks marked 'b' represent substrates incubated for 6 h under assay conditions in the absence of proteinase extract. Tracks marked 'c' represent substrates incubated for 6 h in the presence of proteinase extract.

These included two well-characterised wheat proteins, ribosomal protein L18 [13] and the cytosolic form of phosphoglycerate kinase [14] and two uncharacterised wheat proteins, products of clones chosen by random selection from a cDNA library corresponding to the mRNA content of dry wheat embryos. These clones, designated pWGR2 and pWGR4 encode mRNAs whose abundance was unchanged during the course of wheat embryogenesis, dehydration and subsequent germination, and which are therefore assumed to represent constitutively active genes. Additionally, their cognate mRNAs differ in abundance, the pWGR4 mRNA being ca. 10-fold more abundant than that of pWGR2. The polypeptide products of these clones were 27,000 kDa (pWGR4) and 17,500 kDa (pWGR2).

Finally, we tested the activity of the endopeptidase towards polypeptides whose regulation is largely identical with that of Em. These polypeptides were 'dehydrins' from barley (dhn18-x15287) and maize (dhn3-x15290) [15] whose synthesis, like that of Em, is promoted by ABA and osmotic stress, and whose degradation is triggered upon relief from these stimuli.

The results of these tests are shown in Fig. 4. It is clear that the activity of the proteinase is strongly preferential with respect to the Em polypeptide. No proteolytic products were detected when the constitutively expressed gene products (rpL18, PGK, PWGR2 and pWGR4) were incubated with the 'Fraction 2' proteinase. (Digestion of PGK appeared to result in a reduction in the intensity of the band corresponding to this substrate: this may be due to adventitious exoproteolytic activity derived from the (non-heat-treated) trans-

lation mixture used to prepare this substrate, as no peptides were produced.) A degree of endoproteolysis was apparent when the dehydrin polypeptides were challenged with the proteinase, yielding peptides of apparent M_r ca. 15.5 kDa from the maize substrate, and two peptides of apparent M_r ca. 13 kDa and 11.5 kDa from the barley substrate. However, the extent of proteolysis of these developmentally related polypeptides was very much less than that observed when the Em polypeptide was used as a substrate. The preference of this activity towards the Em polypeptide is reinforced when the quantity of unlabelled Em present in each reaction mixture is considered. Whereas the polypeptide products tested were all synthesised, in vitro, in approximately equal quantities (as estimated by the incorporation of [^{35}S]methionine), the levels of pre-existing unlabelled Em polypeptide in the (wheat-germ) translation extract subsequently supplemented with proteinase were substantially greater than those of the other polypeptides whose radiolabelled forms were similarly challenged. Comparison of the Em stained bands with those corresponding to the rp L18 and pWGR4 translation products indicated that the specific radioactivity of Em subjected to proteolysis was *at least* 100-fold lower than that of any of the other substrates tested.

Seed germination is fuelled by the catabolism of stored reserves which provide sugars and amino acids to the developing seedling. In wheat, and other cereals, these reserves are principally located within the endosperm, where they are mobilised by the action of hydrolytic enzymes secreted by the aleurone layer and by the scutellum of the germinating embryo. Storage proteins are digested by the combinatorial action of different proteinases [10]. These include carboxypeptidases, aminopeptidases and endopeptidases [11] whose activities have been identified by their ability to digest a range of substrates including synthetic peptides, proteins of non-plant origin and the specific proteins found within endosperm storage bodies [10–12,16–19].

By contrast with the mobilisation of endosperm reserves, comparatively little is known of the catabolism of proteins within the germinating embryo. Embryonic proteins which are hydrolysed in a germination-specific manner are of two classes: storage globulins, located within protein bodies [20], and cytosolic albumins. A conspicuous representative of this latter class, the wheat Em polypeptide is distinguished by its very rapid and selective degradation during the first hours of germination. As a first step towards understanding the mechanism by which the Em polypeptide is selectively destabilised during germination, we have identified a cysteine endoproteinase activity exhibiting a marked preference for the Em polypeptide as a substrate.

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