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Sequential expression and differential hormonal regulation of proteolytic activities during germination in *Zea mays* L.

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Abstract. We have characterized the proteolytic activities (proteases in cooperation with carboxypeptidases) involved in the different stages of germination of maize (*Zea mays* L.) grains. A sequential expression of different groups of proteases (aspartic, cysteine, serine and metallo-proteases) with pH optima in the acidic range has been found by using specific protease inhibitors. Pepstatin-sensitive proteolytic activity (aspartic-protease activity) is dominant in resting grains. Germination is accompanied by the appearance of a proteolytic activity which can be enhanced by low-molecular-weight thiol compounds and inhibited by thiol-protease inhibitors, which is indicative of the involvement of cysteine protease(s). This burst of cysteine-protease activity is coincident with the disappearance of the main storage-protein fractions. We conclude from this that cysteine protease(s), with an acid pH optimum, are good candidate(s) for the proteolytic attack of stored protein reserves in maize. After this stage, where cysteine-protease activity is dominant, a period with larger total proteolytic activity starts, coincidentally with the expression of the different types of other proteolytic activities (serine, aspartic and metallo proteases), in addition to the cysteine-protease activity above mentioned. When the development of carboxypeptidase activity during germination was analyzed, the highest activities were found during the earlier and later stages. This result is indicative of a cooperative interaction between carboxypeptidase and endoproteolytic systems in order to obtain a more effective mobilization of storage proteins in germinating maize grains. The phytohormones, gibberellic acid (GA_3) and abscisic acid (ABA) which can stimulate or inhibit, respectively, the total proteolytic activity in extracts from germinating grains, exert a differential effect on the different proteolytic activities here detected.

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Abbreviations: ABA = abscisic acid; DFD = di*iso*propylfluorophosphate; DTT = dithiothreitol; EDTA = ethylenediaminetetraacetic acid; GA_3 = gibberellic acid; PCMBS = p-chloromercuribenzenesulfonic acid; PMSF = phenylmethylsulfonylfluoride; SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Key words: Abscisic acid (protease regulation) – Germination (seed) – Gibberellin (protease regulation) – Protease – Storage protein – *Zea* (germination)

Introduction

The mobilization of storage proteins during grain germination provides a primary source of amino acids to the growing plant. A requirement for a better understanding of this process is the study of the proteolytic enzyme systems involved. Several such studies have been carried out both in monocotyledoneous and dicotyledoneous species and different reports have been published on the relationship between the appearance of protease activity and hydrolysis of either total protein content or storage-protein fractions during germination of a cereal grain (Harvey and Oaks 1974b; Horiguchi and Kitagishi 1976; Fujimaki et al. 1977; Preston and Kruger 1979; Moureaux 1979). Studies on the protease characteristics of crude extracts or partially purified preparations have also been published for various monocotyledoneous species: barley, rice, maize and sorghum (Garg and Virupaksha 1970; Burger 1973; Harvey and Oaks 1974a; Abe et al. 1977; Doi et al. 1980a). Independently of their origin, these enzymes share several properties: they are endopeptidases with an acid pH optimum and display sensitivity to thiol-blocking reagents. In spite of the reported detection of several proteolytic activities in dry grains and germinating cotyledons, their characterization from the enzymological point of view is poor.

The hydrolysis of endosperm storage proteins during the germination of cereal grains can be attributed to the concerted action of various exo- and endopeptidases. Accordingly, high levels of carboxypeptidase activity have been detected and some of these enzymes have been purified from the endosperm of certain germinating cereals (Doi et al. 1980b; Mikola 1983). These carboxypeptidases have high molecular weights ranging between 90000–150000, pH optima around 5.0, sensitivity to di*iso*propylfluorophosphate, and insensitivity to chelating

agents. The relative importance of the endo- and carboxypeptidase activities in germinating cereal grains is variable. Thus, rye, oats, barley and wheat develop high carboxypeptidase activities relative to endopeptidases during germination, whereas maize, shorghum and rice have much lower carboxypeptidase activity (Winspear et al. 1984).

There are many problems that remain unsolved, namely, how many types of proteolytic enzymes are involved in the degradation of storage proteins, what is the temporal and spatial relationship between the appearance of the enzyme activities and the degradation of storage proteins, what kind of proteases are present in resting grains and-or in germinating grains, and how is the expression of these enzymes controlled. Complexity in terms of tissue specificity and hormonal control is associated with the germination process. The present study was undertaken to characterize the pattern of expression of the different proteolytic activities present during germination of maize grains. In particular, the activities present in resting grains, the time course for the appearance of the various proteolytic activities, and the effect of hormones (gibberellic acid and abscisic acid) on their expression were studied.

Material and methods

Plant material and germination. Maize (*Zea mays* L. pure imbred line W64A) grains from plants grown in a greenhouse at Barcelona, Spain, were germinated at 25° C in the dark for 2 d followed by a daily cycle of 15 h illumination for the required time. At all stages of germination, radicle and coleoptile were removed from seedlings, and grains were frozen by immersion in liquid nitrogen. Grains were stored at −70° C.

Maize grains were also germinated in the presence of the hormones gibberellic acid (GA_3; Merck, Darmstadt, FRG) and abscisic acid (ABA; Fluka Chemie AG, Buchs, Switzerland) which were added, at a final concentration of 10 μM, to the germination trays. Fresh hormone solution was added daily.

Preparation of grain extracts and protease assays. Preparation of the crude extracts used for the proteolytic-activity determinations was carried out essentially as described by Jameel et al. (1984) but using 0.05 M 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris)-HCl pH 7.5, 0.05 M NaCl, 4° C, 1 $ml \cdot g^{-1}$ fresh weight of grains as extraction buffer. Protein content of grain extracts was estimated by a modification of the Lowry method (Bensadoum and Weinstein 1976) using bovine serum albumin as standard. For proteolytic-activity determinations, conventional test-tube assays for hydrolytic enzymes as described by Lanahan and Ho (1988) but using a 1-h incubation period and gelatine as substrate were performed. Each assay routinely contained 50 μg of total protein from each crude extract. The proteolytic activities are expressed as micromoles of tyrosine released from the enzyme-substrate mixture during 1 h of incubation or as percentages of control activities. The buffers used for the pH-dependence studies were Na-succinate (pH 3.0–6.5), 1,4-piperazinediethanesulfonic acid (Pipes) (pH 6.0–7.5) and Tris-HCl (pH 7.0–9.0). Gelatine was always dialyzed against the corresponding pH buffer. To determine the effect of thiol compounds on proteolytic activities, extracts were preincubated with β-mercaptoethanol (5 mM), dithiothreitol (DTT; 1 mM) or cysteine (1 mM) for 30 min before the proteolytic-activity assay.

Carboxypeptidase activity was assayed against 1 mM hippuryl-L-phenylalanine or 0.2 mM N-3,2-furylacryloyl-L-phenylalanine-L-phenylalanine (Sigma, Chemical Co., Poole, Dorset, UK) in 1 ml of 0.05 M Na-succinate pH 5.0 at 25° C (Folk and Shirmer 1963; Ho et al. 1979). Aliquots of crude extracts were added directly to the substrate solution. Activities were measured spectrophotometrically and expressed as increase of absorbance per min at 254 nm for hippuryl-L-phenylalanine or decrease of absorbance per min at 330 nm for N-3,2-furylacryloyl-L-phenylalanine-L-phenylalanine. Protease activity for all experiments refers to gelatine hydrolysis while carboxypeptidase activity refers to hydrolysis of N-substituted dipeptides.

Time-course study of storage-protein hydrolysis during germination. An examination of the total protein content from germinating grains allowed us to follow the levels of the different storage-protein fractions at each germination stage. Frozen grains were powdered in the presence of liquid nitrogen and defatted by sucessive acetone/hexane (59:41, v/v) extraction. Proteins were extracted using 0.1 M Tris-HCl pH 6.8, 4% sodium dodecylsulfate (SDS), 10% β-mercaptoethanol, 20% glycerol, 0.02% bromophenol blue as extraction buffer (2 $ml \cdot g^{-1}$ grains) with mild stirring at room temperature for 1 h. Samples were centrifuged (10000 rpm, 15 min) and supernatants were directly analyzed by electrophoresis in 15% polyacrylamide SDS gels (according to Laemmli 1970). Phosphorylase b from rabbit muscle (97.4 kilodaltons, kDa), bovine serum albumin (68 kDa), ovoalbumin (45 kDa), carbonic anhydrase from bovine erythrocytes (30 kDa), soybean trypsin inhibitor (21 kDa) and lysozyme (14.3 kDa) were used as molecular-weight markers (Sigma).

Detection of proteases after electrophoresis. Extract aliquots from different stages of germination, (unless specified, 80 μg protein was used) were subjected to electrophoresis in SDS-polyacrylamide gels (0.1% SDS, 12.5% acrylamide) copolymerized with gelating as described by Jameel et al. (1984). After electrophoresis, SDS was exchanged with Triton X-100 for 90 min at room temperature, and the gel was incubated for 90 min at 37° C in 0.1 M Na-succinate, pH 5.0. After staining with 0.1% amido black in methanol/acetic acid/water (30:10:60, by vol.) and destaining in methanol/acetic acid/H_2O (30:7:63, by vol.) proteases were visualized as clear regions on a dark-blue background. Effect of protease inhibitors on the different bands with proteolytic activity was investigated as described below.

Effect of inhibitors. Protease inhibitors were prepared in the form of concentrated stock solutions as follows: pepstatin A, leupeptin, N-ethylmaleimide, iodoacetic acid, *p*-chloromercuribenzenesulfonic acid (PCMBS) (Sigma), Na_2-ethylenediaminetetraacetic acid (EDTA), 1,10-phenanthroline and $HgCl_2$ (Merck) in water; phenylmethylsulfonylfluoride (PMSF) and di*iso*propylfluorophosphate (DFP) (Sigma) in *iso*propanol. In this later instance, control assays were carried out containing the equivalent concentration of *iso*propanol but in the absence of inhibitor. For studies on the inhibition of the proteolytic activity in crude extracts, all inhibitors were added to extract aliquots and incubated in ice for a period of time between 30 min and 1 h prior to the addition of the substrate. The pH during preincubation with inhibitors was 5.0 except for DFP experiments which were carried out at pH 6.5. This was followed by the estimation of proteinase activity in solution against gelatine (total proteolytic activity) or N-substituted dipeptides (carboxypeptidase activity), or by the electrophoretic detection of proteases. Addition of inhibitors to the Triton X-100 wash and succinate-buffer incubation after gelatine-containing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was checked and found to be unnecessary.

Results

Crude extracts prepared as described under *Material and methods* retained their full proteolytic activity upon standing at 4° C for 24 h. Activity generally decreased

Table 1. Comparison of the effects of different thiol-compounds on the activation of maize protease activities. Crude extracts, previously dialyzed against 0.05 M Na-succinate buffer pH 5.0, were preincubated with or without (control) thiol-reagents at the final concentration indicated for 30 min at 4° C before the assay. Reagents were present at the same concentrations during the incubation period with gelatine. The values obtained without thiol compounds (control values) are arbitrarily set to 100%

Compound tested	Protease activity (% control)	
	Day 0	Day 4
Control (none)	100	100
β-Mercaptoethanol (5 mM)	111	770
Dithiothreitol (1 mM)	100	450
Cysteine (1 mm)	100	450

between 24 and 48 h of storage and practically vanished by 72 h. We found a linear relationship between the amount of crude extract added to the gelatine and the measured protease activity over the range from 5 to 100 µg of protein in crude extracts. The linearity of the carboxypeptidase assay was observed over the range from 5 to 55 µg of protein added to 1 ml of 1 mM substrate (hippuryl-L-phenylalanine). In all experiments described below (protease- and carboxypeptidase-activity assay), the protein content in extract aliquots used for the proteolytic activity determination was within the mentioned linear range.

Previous studies carried out on the characterization of protease activities appearing during grain germination in several species indicate that the activity is enhanced by the presence of β-mercaptoethanol. Accordingly, an examination of the effect of different thiol-compounds, β-mercaptoethanol, DTT and cysteine on the total proteolytic activities of two completely different stages of the germination process, day 0 (resting grains) and day 4, was carried out (Table 1). Results obtained indicate that the proteolytic activities present in resting grains are not affected by thiol-compounds while activities in germinating grains are strongly activated. β-Mercaptoethanol was the most effective of the three thiol-compounds (β-Mercaptoethanol, DTT and cysteine) here tested for their ability to activate the proteolytic enzymes present in extracts prepared from 4-d-germinated grains. The activity of the crude extracts with respect to the controls was almost eightfold higher in the presence of β-Mercaptoethanol and four- to fivefold higher when DTT or cysteine was present.

In order to establish the pH optima for the proteolytic activity during germination, a pH activity profile was obtained from grains germinated for 2, 3, 4 and 5 d (data not shown). Maxima of enzyme activity peaking at pH 5 were found for days 2, 4 and 5 of germination while a maximum of activity at pH 3.5 was detected at day 3 of germination. The existence of proteases in the maize endosperm with pH optima of 3.8 and 3.0 has already been reported (Harvey and Oaks 1974a; Abe et al. 1977).

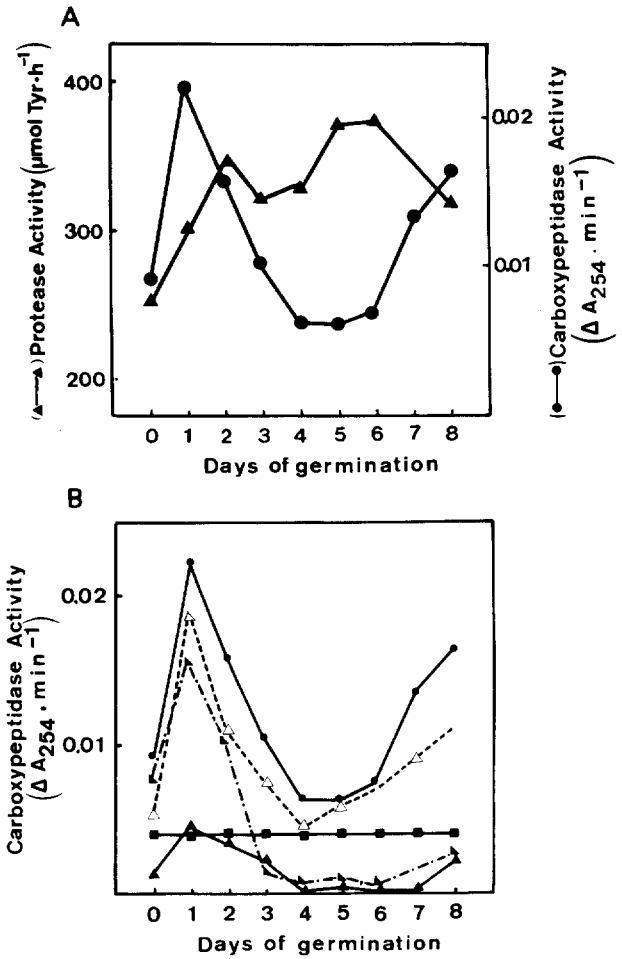

Fig. 1 A, B. Time course for the development of total protease and carboxypeptidase activities during germination of maize grains (**A**) and effect of inhibitors on the carboxypeptidase activity (**B**). **A** Aliquots of fresh extracts containing the same amount of protein for each stage of germination were assayed in the hydrolysis-of-gelatine test for total proteolytic-activity determination (▲—▲; 50 µg protein in each assay) and hydrolysis of hippuryl-phenylalanine for carboxypeptidase activity (●—●; 40 µg protein in each assay). The total proteolytic activity was assayed at pH 5.0 after preincubation of the crude extracts in 5 mM β-mercaptoethanol. Each point represents the mean value for three different germination experiments and three separate preparations of grain extracts for each germination. **B** Extract aliquots (40 µg protein per extract) from resting and germinating grains were preincubated with inhibitors for 30 min at 0° C before assay for carboxypeptidase activity. ●—●, no inhibitor; ■—■, 1 mM DFP; △----△, 5 mM EDTA; (▲—▲, 50 mM EDTA; ▲-·-·-▲, 1 mM PMSF

Time course for the development of total protease and carboxypeptidase activities and mobilization of protein reserves during germination. The increase in the total proteolytic activity in germinating grains is shown in Fig. 1 A. The activity was assayed at pH 5.0 for all stages of germination (although at day 3 the maximum of activity was found at pH 3.5). Proteolytic activity increased with germination time, reaching a maximum at days 5 and 6, and then declined. When the development of the carboxypeptidase activity was analyzed, a different pro-

file was obtained (Fig. 1A). Carboxypeptidase activity was higher during the earlier and later stages of the germination period analyzed, and was maximal at 24 h of imbibition. Carboxypeptidase activity was assayed against two N-substituted dipeptides, (see *Material and methods*) at pH 5.0. Time patterns with both substrates were the same but the activity towards N-3,2-furylacryloyl-L-phenylalanine-L-phenylalanine was only 20% of that for hippuryl-L-phenylalanine under our assay conditions. It has been reported that hydrolysis of zein during germination is accompanied by the formation of free amino acids and, in particular, the liberation rates of phenylalanine and tyrosine are predominant over those of others (Fujimaki et al. 1977). This is the reason why in our routine assays for carboxypeptidase activity we have tested the ability of extracts to liberate phenylalanine from N-substituted dipeptides.

To characterize further the carboxypeptidase activity we investigated its susceptibility to various inhibitors (Fig. 1B). Di*iso*propylfluorophosphate was an effective inhibitor and reduced carboxypeptidase activity to a constant level at all stages of germination. When EDTA was assayed for inhibition, the measured activity was greatly reduced when the inhibitor concentration was increased from 5 mM to 50 mM.

Several acid serine-carboxypeptidases have been characterized and purified from different plant species. However, they are insensitive to chelating agents. Only one neutral metallo-carboxypeptidase has been purified from rice seedlings (Doi et al. 1980c). This enzyme is not affected by DFP but is completely inactivated by EDTA. The carboxypeptidase activity here observed in crude extracts against hippuryl-L-phenylalanine at pH 5.0 probably represents a combination of acid-carboxypeptidase activities, serine and metallo-carboxypeptidases. We have not analyzed neutral-carboxypeptidase activities in germinating maize grains.

The assumption underlying our approach is that those proteolytic activities that are present or increase in level coincidentally with the disappearance of the storage proteins could be involved in their degradation. Accordingly, changes in total protein content of germinating grains are also followed (Fig. 2). Results from our electrophoretic analysis indicate that mobilization and degradation of zeins begins early during germination, the degradation of γ-zein being more rapid than for other zein fractions. These results are in agreement with those reported earlier by Harvey and Oaks (1974b) and recently by our group (Torrent et al. 1989). Figure 2 shows that between days 1 and 5 there was extensive breakdown of the main storage-protein fractions (at day 5, protein bands on SDS-PAGE corresponding to γ- and α-zeins were almost depleted). Previous reports on the degradation of zeins during germination of maize (Fujimaki et al. 1977) indicate that they are degraded to produce low-molecular-weight peptides with no evidence of intermediate polypeptides with electrophoretically different mobilities. It is now then possible to postulate that those proteolytic activities present during the first days of germination (days 1 to 5) could be involved in the proteolysis of the main storage proteins of maize

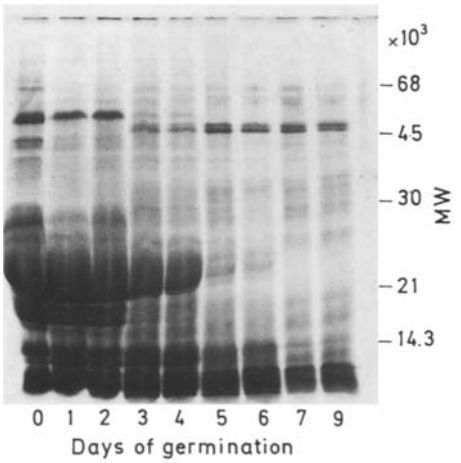

Fig. 2. Total protein contents of extracts prepared from 0- (resting grains) to 9-d-germinated maize grains. The amount of extract in each case is equivalent to 0.04 grains. Major protein bands correspond to the main storage-protein fractions of maize: α-zein (19- and 22-kDa proteins); β-zein (14-kDa proteins); γ-zein (27- and 16-kDa protein); and δ-zein (10-kDa proteins)

grains. Accordingly, inhibitor studies were performed for the characterization of the proteolytic activities present during this period of the germination process (days 1–5) (see below).

Electrophoretic characterization of proteases and the effect of hormones. Gelatine-containing SDS-PAGE was the technique employed to analyze developmental changes in proteases during germination of maize grains between days 1 and 8 of germination. Analysis of gels for protease activity (Fig. 3A) revealed eight distinct proteolytic bands which are visualized as clear areas in the gel following amido-black staining and destaining. We arbitrarily grouped these proteolytic bands into three regions (I, II, III) according to their electrophoretic mobilities. Region I contained five different proteolytic bands (1–5), region II contained two proteolytic bands (6–7) (better seen in Fig. 3B) while just one band (8) was seen in region III. Proteolytic activity 1 was present as early as day 1, proteolytic activities 2 to 5 appeared at day 2, increased until day 5, decreased afterwards and were absent by day 8. At day 2 of germination we detected the appearance of the second region of activity (region II) which remained detectable until day 8. When the same samples were run in gels containing a lower percentage of acrylamide (0.1% SDS, 7% acrylamide), poor resolution among proteolytic bands in region I was obtained. This was probably the consequence of difusion of proteins during the Triton X-100 exchange period (90 min) and subsequent incubation in 0.1 M Na-succinate pH 5.0 (90 min). In addition, on 7% acrylamide gels the blue background after staining became weaker than on 12.5% acrylamide gels and the proteolytic bands were not so well visualized.

The stimulation of the expression of several hydrolytic enzymes in barley aleurone layers by GA_3 and the inhibition of this effect by ABA have been well docu-

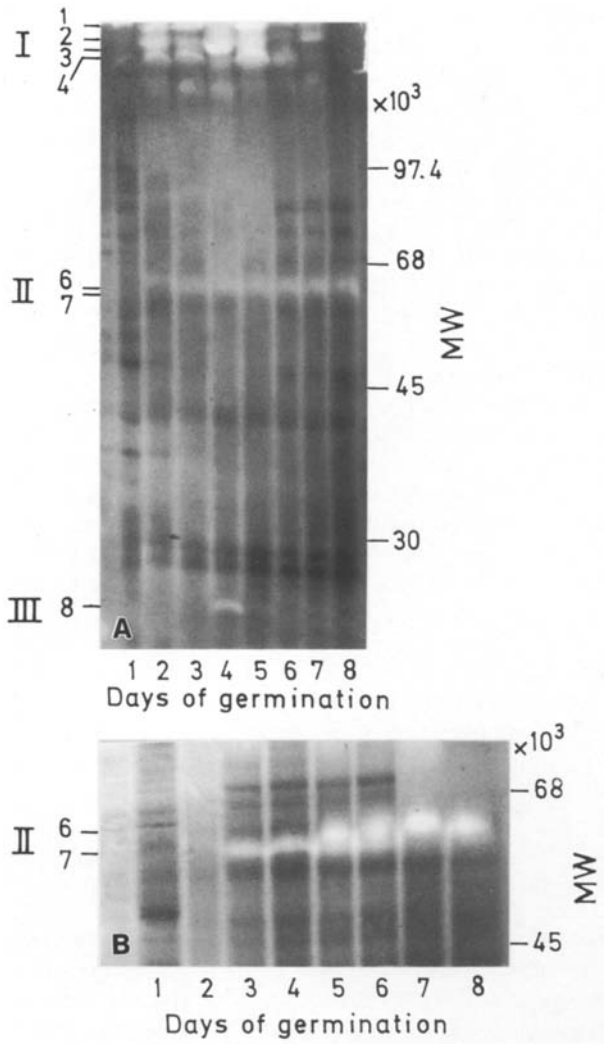

Fig. 3A, B. Analysis of protease activity by PAGE of extracts from maize grains during germination (80 μg protein per assay was used). **A** Gelatine-containing SDS-polyacrylamide gel (0.1% SDS, 12.5% acrylamide) shows three different regions of protease activity (I to III). **B** A gelatine-containing SDS-polyacrylamide gel (0.1% SDS, 10% acrylamide) with greater resolving power reveals two different proteolytic bands in region II. Molecular weights of standard proteins are listed on the right

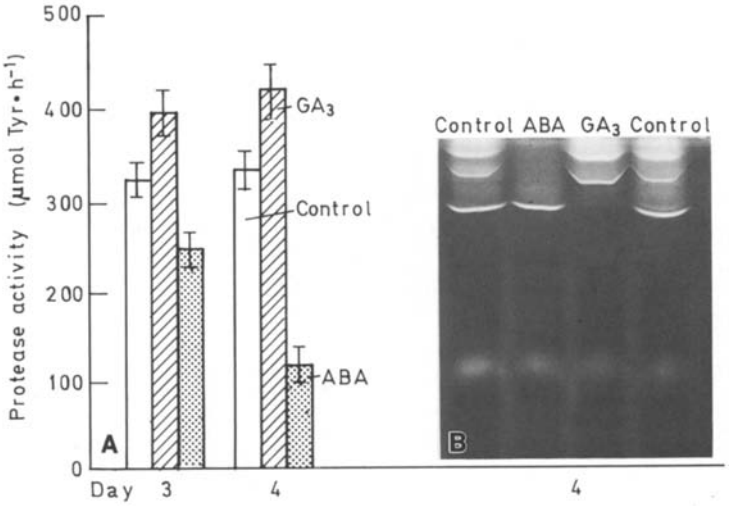

Fig. 4A, B. Effect of hormones (GA_3 and ABA) on the expression of proteolytic activities in germinating maize grains. Grains were germinated for 3 and 4 d with GA_3 or ABA (10 μM each), or without added hormone (*control*). 50 μg protein per assay was used. **A** Total proteolytic activity. Results are the average of three different germination experiments and three separate preparations of grain extract for each germination period. *Arrow bars* indicate maximum and minimum values obtained. **B** Analysis by PAGE of protease activity in extracts prepared from 4-d-germinated grains

mented (Hammerton and Ho 1986; Nolan and Ho 1988; Koehler and Ho 1988). However, in maize, very little information is available. Harvey and Oaks reported in 1974 (Harvey and Oaks 1974c) that degradation of protein reserves in maize was not markedly stimulated by GA_3 but was strongly inhibited by ABA. By analyzing total proteolytic activity, we observed that these hormones caused an increase (GA_3) or decrease (ABA) in the total proteolytic activities at days 3 and 4 of germination, (Fig. 4A) although these changes were not quantitatively large (see day 3). The total stimulatory or inhibitory effect was enhanced from day 3 to day 4 of germination. Qualitative aspects of this hormonal effect are nevertheless important. It is shown in Fig. 4B that either GA_3 or ABA can differentially affect the expression of the various proteolytic activities here detected. This differential effect was observed either at day 3 or day 4 of germination.

Effect of protease inhibitors on the proteolytic activities appearing during the germination of maize grains. The proteolytic activities that appear during the germination process were further characterized by investigating their sensitivities to various inhibitors at each stage of germination. Inhibitors specific for every class of proteases, i.e., DFP and PMSF for serine proteases; leupeptin for cysteine (and some serine) proteases; PCMBS and other thiol-directed reagents (iodoacetic acid, ethylmalemide, $HgCl_2$) for cysteine proteases; pepstatin for aspartic proteases; and EDTA and 1,10-phenanthroline for metalloproteases, were assayed. The effect of protease inhibitors was followed either with gelatine-containing SDS-polyacrylamide gels (Fig. 5) or by the "in vitro" test assay for proteolytic-activity determination in solution (Fig. 6). Figure 5A shows the effect of DFP on the electrophoretic profile of proteolytic activities appearing during germination. Partial inhibition was observed for many of the proteolytic zones present in region I. Di-*iso*prophylfluorophosphate was a particularly effective inhibitor of proteolytic activity at day 4 of germination. In addition, crude extracts from 5-d-germinated grains were also preincubated with the different protease inhibitors (Fig. 5B) and the electrophoretic profile of proteolytic activities was obtained. Sensitivity to these inhibitors was found for the different proteolytic bands: bands 1 and 2 (and probably 6) with leupeptin, band 3 with PMSF, band 4 with EDTA, bands 1, 2 and 5 with DFP. A partial inhibition by pepstatin was probable for bands 1 and 2.

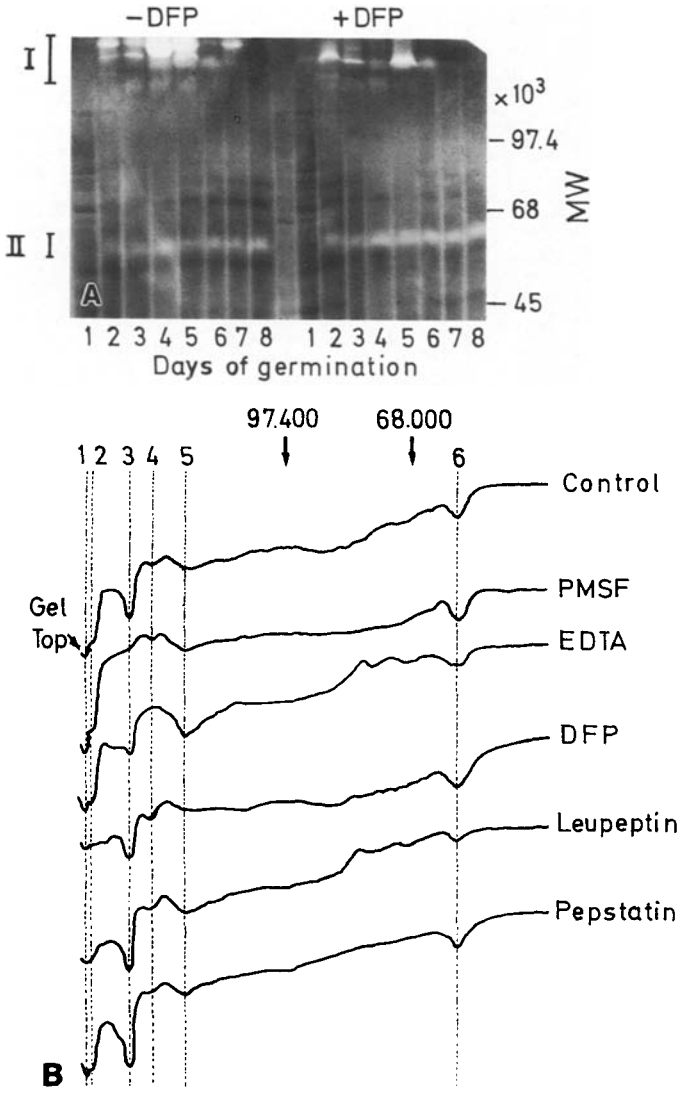

Fig. 5 A, B. Effect of protease inhibitors on the electrophoretic profile of proteolytic activities in germinating maize grains. **A** Effect of DFP on the profile of proteases appearing during germination. Extracts were preincubated with or without 1 mM DFP in ice for 30 min before gelatine-containing SDS-PAGE (0.1% SDS, 12.5% polyacrylamide). **B** Effect of different inhibitors on the proteolytic activities of crude extract from 5-d-germinated grains. Equal aliquots of extracts were preincubated with each inhibitor (2 mM PMSF, 50 mM EDTA, 1 mM DFP, 60 μM leupeptin and 40 μM pepstatin) for 30 min at 0° C and run in parallel in a gelatine-containing SDS-polyacrylamide gel. Densitometric scans of each gel lane are presented. Proteolytic bands (*1–6*) are indicated by *discontinuous vertical lines*

From the analysis of the effect of inhibitors on the total proteolytic activities of extracts prepared from resting (day 0) and germinating grains by the "in vitro" test-tube assay (Fig. 6) several conclusions can be drawn. As shown before (see Fig. 1) extracts prepared from resting grains showed moderate activity in the hydrolysis of gelatine at pH 5.0. However, pepstatin, a powerful and highly selective inhibitor for aspartic-proteases, completely inhibited this activity (Fig. 6A). As germination progressed the percentage of inhibition achieved by pepstatin became progressively lower from day 0 to day 5. Inhibition experiments with leupeptin, were also carried out. When increasing concentrations of inhibitor were assayed, in the presence of β-mercaptoethanol, a progressively greater degree of inhibition was obtained (Fig. 6A). However, when β-mercaptoethanol was not present in the assay (Fig. 6C), protease activity in extracts from 2-, 3- and 4-d-germinated grains was completely inhibited. Later, the inhibitory capacity of leupeptin decreased. The inhibition of protease activity by leupeptin as well as the activation by low-molecular-weight thiol-compounds (β-mercaptoethanol, DTT and cysteine) (Table 1) is indicative of the involvement of cystein-protease(s). More direct evidence came from the inhibition of the proteolytic activities after preincubation in 1 mM PCMBS (inhibitor of SH-enzymes) and other thiol-directed reagents (iodoacetic acid and N-ethylmaleimide) and mercurial reagents ($HgCl_2$). Figure 6C shows the results of inhibition with these thiol-protease-specific reagents. The inhibitory effect of PCMBS, iodoacetic acid and $HgCl_2$ increased as germination progressed. The maximum level of inhibition peaked at day 3 of germination, subsequently decreasing. Proteolytic activity in extracts from 2-, 3- and 4-d-germinated grains was completely inhibited by N-ethylmaleimide. Consequently, proteolytic activities present at days 2, 3 and 4 of germination are particularly sensitive to cysteine-protease inhibitors.

Discussion

The use of specific inhibitors has facilitated the analysis and characterization of the different proteolytic activities involved in the germination of maize grains. Results presented here indicate that the proteolytic system found at each stage of germination shows different properties based on its susceptibility to inhibitors. This would be in agreement with a temporal and sequential pattern for the expression of specific proteolytic activities during grain germination in maize. To start with, proteolytic activities present in resting grains and in given stages of germinating grains are different: aspartic-protease activity or cysteine-protease activity are dominant, respectively. The presence of the pepstatin-sensitive activities in resting and germinating grains has been already reported for other plant seeds, such as rice and Scots pine (Doi et al. 1980a; Salmia 1981). However, in these species the pepstatin-sensitive activity remains at approximately the same level during germination. In addition to the pepstatin-sensitive activity already present in resting grains, there is an important increase in carboxypeptidase activity early during germination. This carboxypeptidase activity presumably would provide free amino acids for the synthesis of the enzymatic machinery required for the mobilization of reserve substances (like hydrolases which are synthesized "de novo" once grain germination has started). Afterwards, a period is found in which cystein-protease activity predominates (days 2, 3 and 4 in our experiments). The period during which cysteine-protease activity is dominant coincides with the disappearance of the main storage-protein fractions. Nevertheless, further studies will be needed to prove a

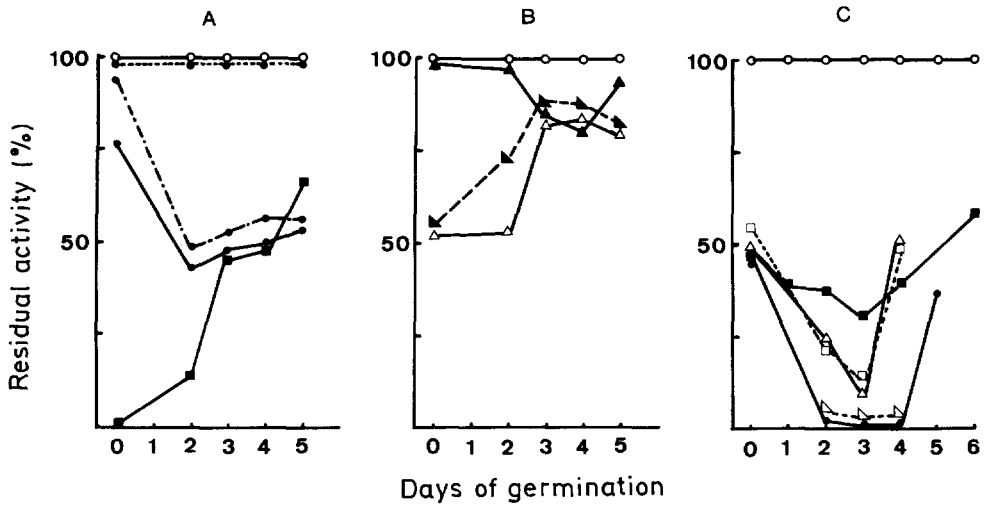

Fig. 6A–C. Effects of inhibitors on the total proteolytic activities of maize grains. Extracts were prepared from resting (day 0) grains and from grains at different stages of germination. Aliquots of extracts (80 μg each) were preincubated with (**A**, **B**) or without (**C**) 5 mM β-mercaptoethanol for 30 min at 0° C. Afterwards, inhibitors were added to extracts and samples were kept in ice for 60 min prior to addition of gelatine. Inhibitor concentrations refer to preincubation and reaction mixtures. Activities are expressed as percentage of activity with no inhibitor. Inhibitors and concentrations used were: **A** ○—○, no inhibitor; ●---●, 1 μM leupeptin; ●-·-·●, 50 μM leupeptin; ●—●, 100 μM leupeptin; ■—■, 100 μM pepstatin; **B** ○—○, no inhibitor; ▲—▲, 1 mM DFP; ▲---▲, 1 mM PMSF; △—△, 50 mM EDTA; **C** ○—○, no inhibitor; ●—●, 100 μM leupeptin; ■—■, 1 mM PCMBS; □---□, 10 mM iodoacetic; △---△, 10 mM ethylmaleimide; △—△, 10 mM $HgCl_2$

causal relation and also to determine if there is just one or several cysteine-proteases, each being specifically involved in the proteolysis of one or more of the different storage-protein types in maize grains.

The increase in total proteolytic activity (days 5 and 6) that follows the stage where cysteine-protease activity is dominant coincides with the appearance of different types of proteases with susceptibilities to inhibitors for all groups of proteases (serine, metal, cysteine and aspartic-proteases). These different proteolytic activities would presumably degrade polypeptide products during the first stage of proteolysis to produce a net degradation of proteins. In addition, further degradation of oligopeptides to amino acids would be produced by the action of carboxypeptidases (and aminopeptidases) in the later stages of germination.

A major problem in the study of proteases acting in cereal grains is the uncommon solubility properties of the major storage proteins. Owing to this problem, investigators have often used artificial protein substrates such as hemoglobin, azocasein or gelatine to assay the proteolytic activities which develop in cereal grains after imbibition. In maize, the major storage proteins are zeins, which are soluble in 70% ethanol (Osborne et al. 1914). It is worth pointing out that a protease with an acidic pH optimum and active against hemoglobin, denatured zein and the β-chain of insulin has been found in maize endosperm (Harvey and Oaks 1974a; Abe et al. 1977; Moureaux 1979).

The majority of studies relating to protein mobilization and the proteolytic activities that may be involved in this process during grain germination have been carried out in dicotyledoneous species, principally legumes. A recent example has been the identification of two different cysteine-proteases (proteases K1 and G1) in germinating soybean (*Glycine max;* Wilson et al. 1988). These two enzymes appear to be involved in the initiation of the proteolysis of two different storage proteins. Likewise, in the mung bean, *Vigna radiata,* the degradation of the major storage globulin, vicilin, seems to be initiated by another cysteine-protease, vicilin peptidohydrolase (Baumgarther and Chripeels 1977). Recently, two proteolytic enzymes, a cysteine protease and a carboxypeptidase, responsible for the breakdown of the main storage protein, 13S globulin, were purified from buckwheat seedlings (*Fagopyrum esculentum* Moench) (Dunaevsky 1989). Furthermore, a cysteine-protease whose synthesis and secretion by aleurone layers is induced by GA_3 has been purified and characterized from barley (Koehler and Ho 1988), one of the most extensively studied cereals. In maize, a cysteine-protease with a pH optimum at 3.0 (using hemoglobin as substrate) has been purified from germinating grains of flint corn (Kóshú cultivar; Abe et al. 1977). We are not aware of other information about the enzyme system responsible for the proteolysis of storage proteins during germination in maize. Variability among published works, particularly about the time of appearance of proteolytic activities in maize (maximum at day 8 of germination) could reflect differences among different breed lines: *Zea mays* hybrid wf9 × 38-11 (Harvey and Oaks 1974a) and W64A (present work). These differences could also be attributed to different endogenous levels of GA_3 in the endosperm tissue of different batches or lines of maize grains.

The present study does not analyze the relative roles of the aleurone layer and scutellum on the synthesis and/or secretion of proteases into the starchy endosperm at different stages of germination. The results described above have been obtained by using whole grains, that is, embryonic axis and scutellum were not removed from

grains. However, we should mention that studies on proteolytic activities were also carried out after removing the embryonic axis and scutellum (data not shown). No differences were observed in these results compared with those obtained with whole grains, either regarding the total proteolytic activity or the electrophoretic pattern of proteolytic activities here presented during maize grain germination.

Results reported here on the effect of hormones on proteolytic activities indicate that although the total proteolytic activity can be stimulated by GA_3 or inhibited by ABA, these hormones cause specific effects on the expression of the individual proteases. Specific genes for proteases could thus be differentially turned on-off by the effect of hormones during germination. Mechanisms by which these differences in hormonal response occur should therefore be different for the different proteases, and remain to be ascertained. Information about how hormones can exert their regulatory roles on gene expression in plants is scarce. Our findings indicating a differential effect of GA_3 and ABA on the expression of individual proteases during germination of maize grains shows that this is a suitable model for studying hormonal regulation processes in plants. Besides, knowledge of the time of appearance and the levels of the different types of proteolytic activities in maize grains in the different stages of germination will facilitate the cloning and characterization of the putative regulatory sequences and a more complete study about factors controlling their expression (i.e. factors which are related to tissue-specific expression and the hormonal regulation of these protease genes). The extent of mobilization of storage-protein reserves may be fine-tuned by regulating the appearance and quantity of the initiating proteases. Finally, the efficiency of the proteolytic enzyme system present in germinating grains could be considered a good measure of grain vigor and so incorporated into regular germination-capability tests.

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