Plant Physiol. (1996) 112: 1211–1217

Characterization of the Endoproteases Appearing during Wheat Grain Development[1]

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The pattern of endoproteolytic activities occurring during wheat (*Triticum aestivum*, cultivar Chinese Spring) grain development was investigated. Total endoprotease activity, assayed in solution with azocasein as a substrate, increased during the early stages of grain development to reach a maximum at 15 d postanthesis that was maintained until the grain was mature. Endoprotease activity was also assayed in gradient polyacrylamide gels co-polymerized with gelatin. The increase in endoproteolytic activity was due to the appearance of up to 18 endoproteolytic bands that were arbitrarily classified into five groups (A, B, C, D, and E). The presence of serine, aspartic, metallo, and, to a lesser extent, thiol proteases in developing wheat grains was demonstrated by the use of class-specific protease inhibitors. The appearance of the different classes of endoproteases during seed development was subject to temporal control; serine proteases were more abundant at early stages and aspartic and metallo proteases were more abundant at later stages. At intermediate stages of development (15–20 d postanthesis), most of the endoproteases were localized in the aleurone, testa, and embryo. The content of acidic thiol proteases was low in the developing starchy endosperm.

The life cycle of the cereal grain comprises two phases, development and germination, which are separated by a period of dormancy (Rock and Quatrano, 1995). In wheat (*Triticum aestivum*), as in other cereals, the endosperm differentiates into two tissues during caryopsis development, the aleurone layer and the starchy endosperm (Morrison et al., 1975, 1978). Recently, Bosnes et al. (1992) reported a detailed description of endosperm development in barley grains that, according to these authors, is subdivided into four main stages: syncytial, cellularization, differentiation, and maturation. During maturation storage material accumulates in the starchy endosperm, and this reserve constitutes the major nitrogen and carbon source for seedling growth following germination. Therefore, during the caryopsis life cycle the starchy endosperm undergoes antagonistic processes: the accumulation of storage material during development and its mobilization during seedling growth. Hormones play an important role in the control of these processes. ABA, a plant hormone that prevents germination (Wang et al., 1995), reaches maximum levels at the maturation stage when storage material is synthesized (King, 1975; Rock and Quatrano, 1995); the level of GA, however, is low at this stage. During germination, when ABA levels are low, GA is released from the embryo to the starchy endosperm, where it activates the synthesis and secretion of hydrolytic enzymes by the aleurone layer (Fincher, 1989).

Among the hydrolases produced by the aleurone layer during germination, proteases in different cereals have received much attention (Rastogi and Oaks, 1986; San Segundo et al., 1990; Wrobel and Jones, 1992; Mitsuhashi and Oaks, 1994; Domínguez and Cejudo, 1995); however, very little is known about proteolysis during grain development. Although it is generally assumed that developing grains do not contain active reserve-mobilizing enzymes, several reports suggest that at some stage of development proteases may be synthesized. Mature grains of barley (Sarkkinen et al., 1992; Wrobel and Jones, 1992), rice (Doi et al., 1980), maize (Mitsuhashi and Oaks, 1994), and wheat (Morris et al., 1985; Belozersky et al., 1989) contain proteases.

To our knowledge, no description of the pattern of endoproteases in developing cereal grains has yet been reported. Our goal in this study was to analyze the endoproteases that appear during grain development in wheat. For that purpose we have taken advantage of a method based on gradient SDS-PAGE co-polymerized with gelatin, an artificial substrate for plant proteases, which allowed a high resolution for analyzing the pattern of endoproteases during germination (Domínguez and Cejudo, 1995). Our results show the presence of significant endoproteolytic activity in developing wheat grains. In contrast to germination, this activity is due mostly to the appearance of neutral endoproteases, although acidic aspartic and thiol proteases are also detected. Neutral endoproteases are localized in the aleurone-embryo-testa fraction at intermediate stages of development and are almost absent from the starchy endosperm. The possible role of the hormones ABA and GA in the control of this pattern of expression is discussed.

[1] This research was supported by grant PB92–0675 from Dirección General de Investigación Científica y Técnica, Ministerio de Educación y Ciencia, and group no. 3213 from Junta de Andalucía, Spain.

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Abbreviations: dpa, days postanthesis; IA, iodoacetate; pHMB, *p*-hydroxymercuribenzoate.

MATERIALS AND METHODS

Wheat (*Triticum aestivum* cv Chinese Spring) grains were sterilized in 3% (v/v) NaOCl for 20 min, washed once with sterile water and 0.01 M HCl, and then thoroughly washed again with sterile water. Sterilized grains were germinated for 2 d at 25°C on filter paper soaked with water and then maintained at 4°C for 2 d. The germinated seeds were cultivated in a greenhouse under a 16-h day/8-h night cycle at 22 to 25°C. Under these growth conditions the development was completed about 40 dpa (Fig. 1A). At different stages of development grains were harvested, and the length and chlorophyll and gliadin contents were determined. A typical time course showing the variation of these parameters during wheat grain development under the growth conditions used in this work is shown in Figure 1B. Grains harvested at different stages of development were frozen in liquid nitrogen and kept at −70°C until needed. Dissection to separate the aleurone-testa-embryo and starchy endosperm-subaleurone fractions was performed as described by Bosnes et al. (1992).

Protein and Chlorophyll Extraction

The differential extraction of wheat grain proteins was carried out as described by Kobrehel et al. (1992). Albumin/globulin and gliadin fractions were extracted sequentially from liquid nitrogen-frozen grains at different stages of development as follows: 50 mM Tris-HCl, pH 7.5, for 20 min, albumin/globulin fraction and then the gliadin fraction in 70% (v/v) ethanol for 2 h. All extractions were carried out at 25°C.

Extracts for protease assays were obtained from homogenized grains or dissected tissues by extraction in 50 mM sodium acetate, pH 4.7, and supplemented with 2 mM Cys and 1 mM EDTA as described by Domínguez and Cejudo (1995). Protein determinations were carried out by the Bradford method (Bradford, 1976) using a kit (Bio-Rad).

Chlorophyll extraction and determination were carried out by the method of Arnon (1949).

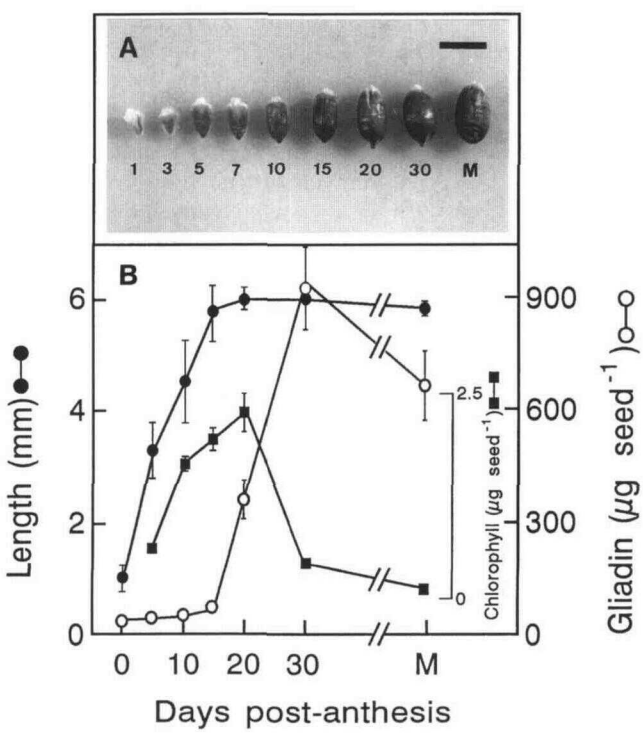

Figure 1. Time course of wheat grain development. A, Grains harvested at different dpa. B, Grain length (●), chlorophyll content (■), and gliadin content (○). M, Mature grains. Bar, 5 mm.

Endoprotease Activity Assay in Solution

Endoprotease activity was determined using a modification of the method described by Holwerda and Rogers (1992). Up to 50 μL of crude extract was incubated with 100 μL of 0.1% (v/v) Brij 35, 300 μL of 100 mM sodium acetate, pH 5.0, and 50 μL of 6% (w/v) azocasein (Sigma). Assays that were linear over at least 5 h were carried out at 30°C for 3 h and then stopped by the addition of 200 μL of 10% (v/v) TCA. After the removal of precipitated protein by centrifugation (13,000 rpm for 15 min at room temperature) the A_{366} of the supernatant was determined. Activities are expressed as micrograms of azocasein hydrolyzed per minute per seed. To analyze the effect of inhibitors on proteolytic activities, assays were carried out in the presence of different class-specific inhibitors at the following concentrations: 50 mM EDTA for metalloproteases, 0.1 mg mL^{-1} pepstatin A for aspartic proteases, 10 mM PMSF for Ser proteases, and 10 mM IA or 1 mM pHMB for thiol proteases.

Endoprotease Activity Assay in Gel

Endoproteases were detected in gradient (4–15% polyacrylamide) SDS-PAGE co-polymerized with 0.1% (w/v) gelatin, as previously described (Domínguez and Cejudo, 1995). Extracts (up to 100 μg of protein) were subjected to electrophoresis. After electrophoresis SDS was removed by incubating gels in 2% (w/v) Triton X-100 (Sigma) for 30 min at room temperature. Gels were then incubated overnight at 40°C in 50 mM sodium acetate, pH 4.0, or in 50 mM sodium phosphate, pH 6.5. To identify acidic endoproteases, a double amount of extract (up to 200 μg of protein) was subjected to electrophoresis and the activity assay was carried out for 24 h. When indicated, the thiol reagent β-mercaptoethanol was added at a final concentration of 10 mM. Proteins used as molecular mass standards were: myosin, 200 kD; β-galactosidase, 116 kD; phosphorylase b, 97 kD; serum albumin, 66 kD; ovalbumin, 45 kD; carbonic anhydrase, 31 kD; and soybean trypsin inhibitor, 21 kD. All of these standards were purchased from Bio-Rad. To analyze the effect of inhibitors on endoproteolytic activities in gel, after electrophoresis and SDS-Triton X-100 exchange, gels were incubated in the presence of inhibitors at the above-described concentrations.

RESULTS

Proteolytic Activity during Seed Development

Extracts prepared from wheat grains at different stages of development showed endoproteolytic activity when azocasein was used as a substrate (Fig. 2). A sustained increase in activity was observed during the first half of development (0–15 dpa), a period coincidental with the increase in grain length (Fig. 1). For comparison, the evolution of the content of gliadin is also shown in Figure 2. The synthesis of storage proteins took place during the second half of the development process (15–30 dpa); endoproteolytic activity did not increase further during this time. The decreased amount of gliadin detected in mature grains was probably due to less efficient extraction of these proteins when grains were completely dry.

To characterize the endoproteases that appeared during the development of wheat, extracts were analyzed by electrophoresis in gradient polyacrylamide gels co-polymerized with gelatin. Assays for endoproteases in gel were carried out at pH 6.5 (Fig. 3A) and pH 4.0 (Fig. 3B). The increase in proteolytic activity when assayed in solution (Fig. 2) was due to an increase in the activity of endoproteases appearing in the grain as development proceeded. These endoproteases showed more activity at pH 6.5 than at pH 4.0; therefore, most of the endoproteases in developing kernels are neutral. These endoproteolytic bands, clearly distinguished at pH 4.0 (Fig. 3B), were arbitrarily classified into four groups: group A, containing three endoproteases with molecular masses greater than 116 kD, readily detected at 15 dpa, and more abundant at late stages of development (20–30 dpa); group B, with molecular masses between 97 and 116 kD. One of the endoproteases of this group (termed B1 in Fig. 3A) was present throughout the development process, although it

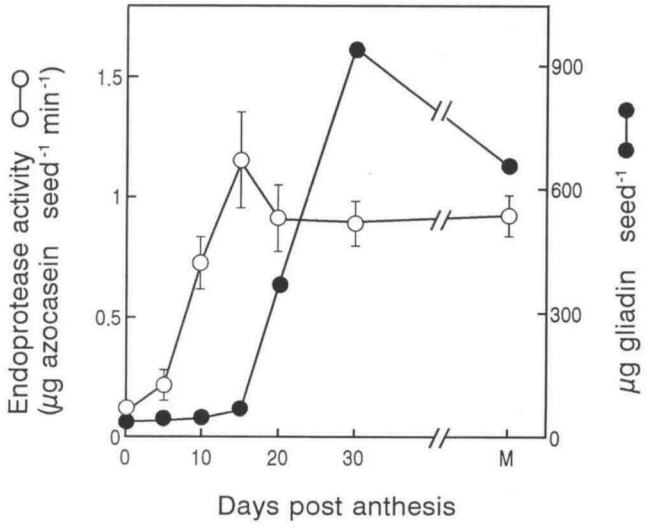

Figure 2. Time course of endoproteolytic activity and gliadin content during development of wheat grains. Aliquots from extracts of grains at different stages of development were assayed for hydrolysis of azocasein (○). The gliadin content (●) is as in Figure 1. Activity values are averages ± SE from three independent assays.

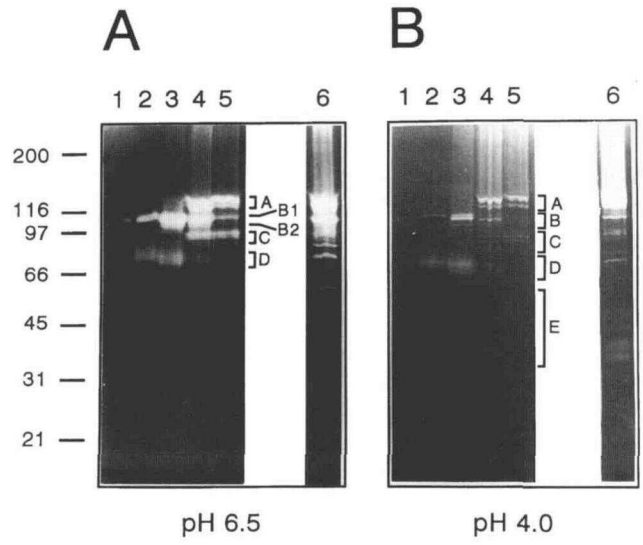

Figure 3. Analysis of endoprotease activities on gradient polyacrylamide gels co-polymerized with gelatin. The same amount of protein (100 μg) from grains harvested at different dpa was loaded per track. After electrophoresis an activity assay was carried out as follows: A, overnight incubation at 40°C in 50 mM sodium phosphate, pH 6.5; B, overnight incubation at 40°C in 50 mM sodium acetate, pH 4.0. Both buffers were supplemented with 10 mM β-mercaptoethanol. Extracts were prepared from developing grains at 5 dpa (lane 1), 10 dpa (lane 2), 15 dpa (lane 3), 20 dpa (lane 4), and 30 dpa (lane 5). An extract from 5-d-germinated grains was also analyzed (lane 6). Molecular masses of standard proteins are marked on the left. Endoproteases were arbitrarily classified into groups A to E as indicated.

was more abundant at intermediate stages (10–20 dpa). Another endoprotease of this group (B2) was detected only at intermediate stages (15–20 dpa); group C, three endoproteases with molecular masses between 85 and 97 kD, appearing only at late stages (20–30 dpa); group D, with molecular masses between 66 and 85 kD and present only at early stages of development (5–20 dpa). For comparison, an extract from 5-d-germinated grains was analyzed (Fig. 3, lanes 6). The apparent high activity at the top of these lanes was due to individual endoproteolytic bands that were identified when assays were carried out for 45 min (data not shown, Domínguez and Cejudo, 1995). It is remarkable that most of the neutral endoproteolytic bands of developing grains showed an electrophoretic mobility coincident with endoproteases detected in germinating grains. However, when the activity was assayed at pH 4.0, the acidic endoproteases characteristic of germinating grains (Fig. 3B, lane 6) were not detectable in developing grains (Fig. 3B, group E). A search for acid endoproteases was carried out by analyzing double amounts of crude extracts and increasing the incubation time for the activity assay (see "Materials and Methods"). Under these conditions up to six new endoproteolytic bands were detected at pH 4.0 in extracts from 15 and 20 dpa (group E). These endoproteases showed the same electrophoretic mobility as thiol proteases of germinating grains (Fig. 6A, see below).

Effect of Class-Specific Inhibitors on the Endoproteolytic Activities of Developing Kernels

The effect of class-specific inhibitors on the endoproteolytic activities of extracts obtained from grains at different stages of development was tested and the results are presented in Figure 4. EDTA, an inhibitor of metalloproteases, produced increasing inhibition in extracts from grains at late stages of development (after 15 dpa), showing the presence of metalloproteases during this period and in mature grains (Fig. 4A). Pepstatin A, an inhibitor of aspartic proteases, was effective from 10 dpa (Fig. 4B). This effect was due to the inhibitor, since methanol, the solvent used for pepstatin A, did not affect endoprotease activity. These results show the presence of aspartic proteases at early stages, even though these endoproteases reached a maximum level during the second half of development (after 10 dpa). Aspartic endoproteases were also abundant in mature kernels. The presence of Ser proteases was tested using PMSF as an inhibitor (Fig. 4C). A high inhibitory effect was observed during the early stages of development and decreased at later stages. This effect was also due to the inhibitor, since isopropanol, the solvent used for PMSF, had no effect on the endoproteolytic activity. These results show the presence of high amounts of Ser proteases early in development that decreased as the process was completed. In mature seeds the content of these endoproteases was very low. The presence of thiol proteases was tested using IA and pHMB (Fig. 4D). No inhibition was observed in the presence of IA, whereas pHMB showed a clear inhibitory effect at early and intermediate stages that decreased at later stages. This pattern was very similar to that obtained with PMSF (Fig. 3C). We conclude from these data that there is a very low level of thiol proteases in developing wheat grains, as shown by the lack of inhibitory effect of IA. The effect of pHMB is most probably due to inhibition of Ser proteases that are abundant at these stages. According to Ryan and Walker-Simmons (1981) the Ser proteases may be inhibited by PMSF and pHMB but not by IA.

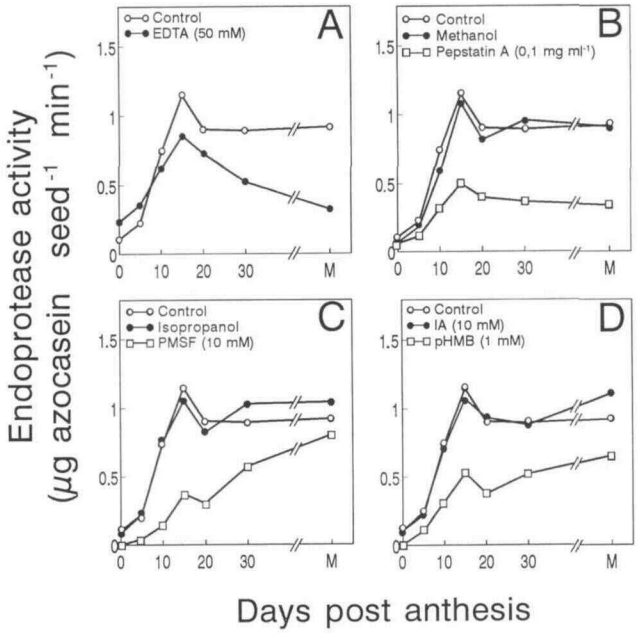

Figure 4. Effect of class-specific protease inhibitors on endoprotease activity of developing grains. Activity assays were carried out in solution with azocasein as a substrate in the absence (control) or presence of inhibitors as indicated. Methanol and isopropanol were the solvents for pepstatin A and PMSF, respectively. Results are representative of at least three independent experiments. M, Mature grains.

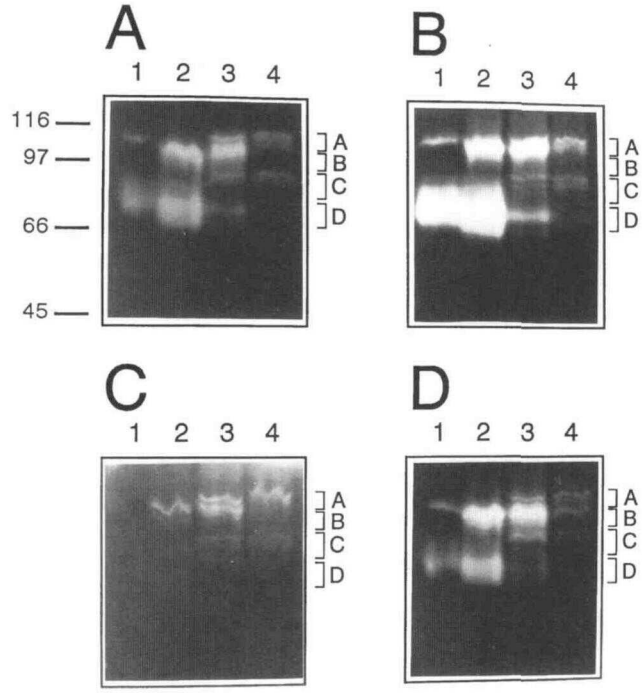

Figure 5. Effect of class-specific inhibitors on endoprotease activity in gel during grain development. Extracts (100 μg of protein) were electrophoresed in gradient polyacrylamide gel co-polymerized with gelatin. Assays were carried out by overnight incubation at 40°C in 50 mM sodium phosphate, pH 6.5, in the absence of inhibitors (A) or in the presence of 50 mM EDTA (B), 10 mM PMSF (C), or 1 mM pHMB (D). Extracts were prepared from grains harvested at 10 dpa (lane 1), 15 dpa (lane 2), 20 dpa (lane 3), and 30 dpa (lane 4). Molecular masses of the standard proteins are indicated on the left. The different groups of endoproteases are marked on the right.

The effect of class-specific inhibitors was also tested on endoprotease assays in gel (Fig. 5). As mentioned above, when the activity assay was carried out in solution with azocasein as a substrate, EDTA exerted a clear inhibitory effect at later stages of development. However, none of the endoproteases detected in gel assays with gelatin as a substrate were inhibited by EDTA (Fig. 5B), which may indicate that gelatin is a poor substrate for metalloproteases. Surprisingly, EDTA showed a general activating effect on most of the endoproteases present in developing kernels. The reason for this activating effect is not yet understood. PMSF caused a complete inhibition of the group D endoproteases and a partial inhibition of the group B and C endoproteases (Fig. 5C). pHMB exerted a partial inhibition of group C and, to a lesser extent, of

group D endoproteases. Based on these data, we conclude that endoproteases of groups B, C, and D are Ser proteases. The different responses of these endoproteases to inhibition by pHMB and PMSF suggest the presence of different groups of Ser proteases in developing wheat seeds. In agreement with the above-described results with azocasein as a substrate, it is clear that most of the endoproteases present in wheat grains during the first half of development are Ser proteases.

As anticipated, no acidic endoproteases were detected when extracts from developing wheat grains were analyzed by SDS-PAGE co-polymerized with gelatin (corresponding to group E in Fig. 3B). However, when the amount of extract analyzed and the incubation time of the activity assay were increased (see "Materials and Methods"), up to six endoproteolytic bands with molecular masses ranging from 40 to 60 kD were detected in extracts from 15 dpa (Fig. 6). The activities of these acidic endoproteases were stimulated by the thiol reagent β-mercaptoethanol (compare lanes 1 and 2 in Fig. 6A) and totally inhibited by either IA or pHMB (Fig. 6B, lanes 2 and 3), indicating that these bands corresponded to thiol proteases. The data showing inhibition with pepstatin A (Fig. 4B) establish the presence of aspartic proteases at later stages of development. Unfortunately, gelatin is a poor substrate for this class of endoproteases (Wrobel and Jones, 1992) and, therefore, they were not detected with the protease assay used in this study. Group A endoproteases were not significantly affected by any of the inhibitors used in this study.

Localization of Endoproteases in Wheat Developing Grains

The analysis of endoproteases in developing grains just described reveals the presence of significant endoproteolytic activity throughout the process of grain development. Since gliadin and glutenin, the wheat storage proteins, are synthesized at intermediate and late stages of development and accumulate in the developing starchy endosperm, we determined the localization of endoproteases in developing kernels to test for the presence of endoproteases in the starchy endosperm. For this purpose, grains at intermediate stages of development (15 and 20 dpa) were dissected in two fractions, aleurone-testa-embryo and starchy endosperm-subaleurone, and their endoprotease content was analyzed. Figure 7 shows that most of the neutral endoproteases of developing grains were localized in the aleurone-testa-embryo (Fig. 7A). It is remarkable, however, that the acidic thiol proteases of group E, which were detected at pH 4.0 but not at pH 6.5 (Fig. 7B), were localized in the starchy endosperm-subaleurone fraction. Since at these stages of development the pH of the starchy endosperm is neutral (Macnicol and Jacobsen, 1992), these thiol proteases are probably inactive in vivo.

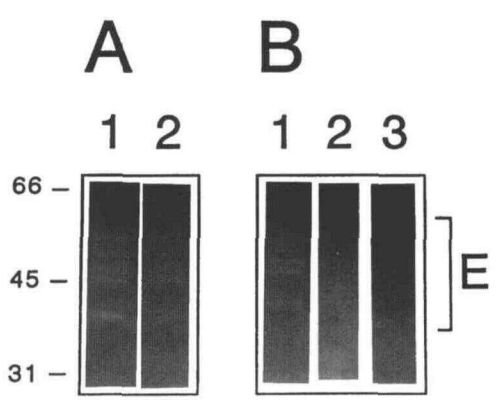

Figure 6. Characterization of group E endoproteases. Extracts (200 μg of protein) were electrophoresed in gradient polyacrylamide gels co-polymerized with gelatin. A, Assays were carried out by 24-h incubation at 40°C in sodium acetate, pH 4.0, in the presence of 10 mM β-mercaptoethanol (lane 1) or in the absence of thiol reagents (lane 2). B, Effect of thiol protease inhibitors. Assays were carried out in the absence of inhibitors (lane 1) or in the presence of 10 mM IA (lane 2) or 1 mM pHMB (lane 3). Molecular masses of the standard proteins are indicated on the left.

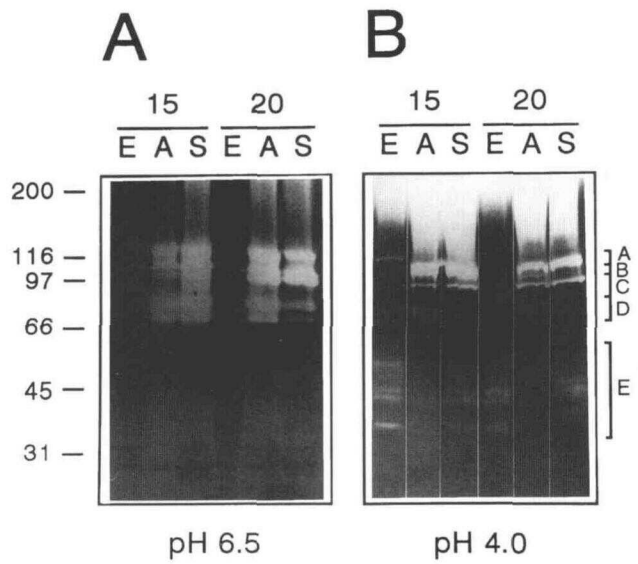

Figure 7. Localization of endoproteases in developing grains. Grains at the indicated stages of development (15 and 20 dpa) were dissected and extracts were prepared from starchy endosperm-subaleurone (E lanes) and aleurone-testa-embryo (A lanes). Extracts from whole grains were also analyzed (S lanes). Activity assays (200 μg of protein extract, 24-h incubation at 40°C) were carried out in 50 mM sodium phosphate, pH 6.5 (A), and 50 mM sodium acetate, pH 4.0 (B). Molecular masses of the standard proteins are indicated on the left. The different groups of endoproteases are marked on the right.

DISCUSSION

Two different phases, development and germination, are distinguished in the life cycle of the cereal grains and require the expression of separate and different sets of genes. Storage materials such as starch, proteins, or lipids accumulate in cells that differentiate during development to form the starchy endosperm. These storage materials are hydrolyzed during germination, providing the initial nutrients for seedling growth. On the basis of this view of the life cycle of the cereal grain, it is assumed that the expression of genes coding for hydrolytic enzymes such as amy-

lases or proteases takes place during the germinative but not during the developmental phase. However, the data presented in this study show a significant amount of endoproteases in developing wheat grains.

The use of class-specific protease inhibitors has shown that developing wheat grains contain all four classes of proteases. During development a temporal pattern of appearance of the different endoproteases is detected. Early after anthesis, the grain contains almost exclusively Ser proteases. At intermediate stages, when the content of Ser proteases decreases, the content of aspartic proteases increase and a very small amount of thiol proteases appears transiently. Aspartic protease and metalloprotease are abundant at later stages of development. The four classes of endoproteases are detected during germination as well. A major difference between the two phases is that most endoproteases of developing kernels are neutral, whereas in germinating kernels similar levels of neutral and acidic activity are detected (Domínguez and Cejudo, 1995). In addition, a group of GA-responsive thiol proteases that are very abundant in the starchy endosperm of germinating grains (Domínguez and Cejudo, 1995) are present at very low levels in developing grains.

The increase in total endoproteolytic activity observed during the first half of grain development (0–15 dpa) is due to the appearance of two groups of Ser proteases, which we have called groups B and D. No other endoprotease class is detected at the early stages of grain development. The presence of Ser proteases has been described in the fruits of the tropical plant *Maclura pomifera* (Rudenskaya et al., 1995) and in mature soybean seeds (Morita et al., 1994). To our knowledge, this is the first report describing Ser proteases in developing cereal grains. Endoproteases with the same molecular masses are also present in germinating seeds, in which they are localized in the scutellum (Domínguez and Cejudo, 1995). It is interesting to note that in either developing or germinating grains these endoproteases are almost absent from the starchy endosperm. The localization of Ser proteases in developing and germinating embryos suggests that their physiological role is in protein metabolism (processing or turnover) rather than in storage-protein degradation.

Both metalloproteases and aspartic proteases appear at later stages of grain development. The significant inhibition exerted by EDTA shows the presence of metalloproteases in mature wheat grains. Metalloproteases are also detected in mature grains of buckwheat (Belozersky et al., 1990; Elpidina et al., 1991) but not in barley (Wrobel and Jones, 1992). Since in buckwheat seeds metalloproteases are localized in the protein bodies, Elpidina et al. (1991) proposed a role for them in the initial limited proteolysis of storage proteins early after imbibition. Aspartic endoproteases are present in mature seeds of wheat (Morris et al., 1985; Belozersky et al., 1989) and barley (Sarkkinen et al., 1992). A barley aspartic protease, HvAp, which has been well characterized (Tormakangas et al., 1994), is widely distributed in both developing and germinating grains but is absent from the starchy endosperm. In addition, aspartic endoproteases do not hydrolyze storage proteins. These data suggest that aspartic endoproteases are not implicated in starchy endosperm mobilization following germination.

Under specific assay conditions, we have identified up to six endoproteolytic bands that show enhanced activity in the presence of thiol reagents and are completely inhibited by IA and pHMB. Based on these data we conclude that these bands correspond to thiol proteases. This class of proteases is very abundant during germination (Domínguez and Cejudo, 1995) and the cDNAs for some of them have been cloned. Northern blot analysis has shown that some thiol proteases are synthesized in aleurone cells in response to GA (Koheler and Ho, 1990; Cejudo et al., 1992). At intermediate stages of development (15 and 20 dpa) the thiol proteases are localized in the starchy endosperm, where the synthesis and accumulation of gliadin and glutenin are also occurring at this time. Therefore, a mechanism (probably hormonal control or specific inhibitors) may exist to prevent full expression and activity of these enzymes. In germinating grains the synthesis and secretion of some thiol proteases from the aleurone layer to the starchy endosperm is enhanced by exogenously added GA_3, and this effect is counteracted by ABA (Nolan and Ho, 1988; Cejudo et al., 1992; Domínguez and Cejudo, 1995). The accumulation of storage proteins in developing grains takes place at a time of decreasing GA levels and maximum levels of ABA (King, 1975; Rock and Quatrano, 1995). In addition, it has been shown that developing aleurone cells are insensitive to treatment with GA_3 unless they are subjected to dehydration (Evans et al., 1975; Armstrong et al., 1982; Norman et al., 1982; Cornford et al., 1986). Therefore, the level of hormones at these stages of grain development (high ABA, low GA) and the insensitivity of aleurone cells to GA may explain the low content of thiol proteases in developing starchy endosperm compared with germinating grains.

ACKNOWLEDGMENTS

We wish to thank C. Gotor for critical reading of the manuscript. The photography work of M.J. Cubas is deeply appreciated.

Received May 14, 1996; accepted August 13, 1996.

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