

Characterization of Oat Endoproteinases that Hydrolyze Oat Globulins

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ABSTRACT

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During the germination of oats, the major seed storage proteins (globulins) are hydrolyzed by endoproteinases. We have used two methods to characterize these endoproteinases. A qualitative PAGE method that used oat globulins as gel-incorporated substrates was used to determine which enzymes hydrolyzed the globulins. The proteolytic hydrolysis products were studied by hydrolyzing the globulins in vitro with the endoproteinases and analyzing the products by SDS-PAGE. Class-specific proteinase inhibitors were used to show that the globulin hydrolyzing enzymes were cysteine-class proteinases. The proteinases were active at pH 3.8. Using the gel analysis method, a little activity was present at the beginning of

seed germination, but the major activity only appeared on the sixth day of germination. Extracts from four-day germinated oats contained cysteine proteinases that hydrolyzed the globulins in vitro to form a polypeptide of intermediate size (MW ≈34,500). Cysteine proteases from an eight-day germinated sample totally hydrolyzed the globulins in <1 hr. Very little hydrolysis occurred at pH 6.2, the pH of germinated oats endosperm tissue. The fact that hydrolysis occurred quickly at pH 3.8 implies that there is probably pH compartmentalization within the endosperm, with some areas of the seed having a low pH value where the globulins can be degraded.

Seeds provide the nutrients that are needed to maintain the requirements of developing plantlets until they become established as photosynthetic autotrophs. The reserve proteins and carbohydrates of the seeds are situated in the endosperm and these insoluble storage products must be hydrolyzed into soluble forms that can be transported to the embryo for utilization.

Although the proteolytic system of the germinating oat seed has not been studied in detail, some data related to this system are available. Donhowe and Peterson (1983) showed that the protein bodies of resting oat seed aleurone layers contained casein-hydrolyzing activity that operated at pH 5–6, but that the protein bodies of the starchy endosperm did not. Sutcliffe and Baset (1973) showed that there was pH 8 active casein hydrolyzing activity in germinating oats and that this activity increased quickly during the first two days of germination, after which it continued increasing, but at a lower rate. By definition, germination is complete when the primary root emerges (Bewley and Black 1994). However in this report, and in keeping with the way the word is used in the malting industry, the “germination” period also includes the time during which the early plantlet growth phase occurs.

In contrast to these few studies of the proteolysis that occurs during germination of oats, numerous reports have been made that relate to the proteolytic activities of germinating barley, and several barley cysteine and aspartic proteinases have been purified and characterized. The hydrolytic specificities of several cysteine proteinases have been reported and their appearances during germination have been explored. A malted barley 30-kDa cysteine proteinase that readily hydrolyzed hordeins, the major barley storage proteins, in vitro was purified to homogeneity by Poulle and Jones (1988). Similar hordein-hydrolyzing proteases have been reported by Koehler and Ho (1990) and Phillips and Wallace (1989).

One barley seed aspartic proteinase has been purified (Sarkinen et al 1992), and its hydrolytic specificity (Kervinen et al 1993) and its location in the seed (Tormakangas et al 1994) have been determined. Because it did not degrade storage globulins (Kervinen et al 1994) but did process pro barley lectin (Runeberg-Roos et al 1994), it may function as a regulatory enzyme in the seed. Partially purified malt aspartic proteinases did not degrade hordein preparation but did hydrolyze barley chloroform-methanol soluble proteins.

The presence of serine and metalloproteinases in germinated barley have been clearly established (Zhang and Jones 1995), although no purified enzymes of either of these classes have been characterized yet. In barley, the cysteine proteinases apparently hydrolyze the bulk of the hordeins, which are the major endosperm storage proteins.

In oats, the major storage proteins are globulins (salt-soluble proteins), while those of the other cereals such as wheat, barley, maize, and rye are generally the alcohol-soluble prolamins. Oats do contain prolamins (avenins), but these are present in smaller quantities than the globulins (Peterson and Brinegar 1986). The oat globulins are heterohexamers with two different subunits: alpha (MW 32,500–37,500) and beta (MW 22,000–24,000). The amount of oat globulins decreased during germination, and only negligible quantities were present after five days of germination (Kim et al 1979). Radioimmunoassays have shown that 75% of the oat proteins are globulins (Colyer and Luthe 1984). According to Shotwell et al (1988), the oat 12S globulins are 70% homologous to the rice storage globulins (glutelins), 38% homologous to pea, and 31% homologous to soy storage globulins. In flowering plants, the 11S globulins are common storage proteins and their hydrolysis during germination is probably initiated by a cysteine proteinase that appears during the early stages of germination (Shutov and Vaintraub 1987).

We showed earlier (Mikola and Jones 2000) that serine and metalloproteinases are the predominant pH 6.2 active gelatin hydrolyzing enzymes of four-day germinated oats. The pH of the germinating oat endosperm tissue is 6.2. Furthermore, we showed that the azogelatinase activity of germinated oats increased in the presence of a mixture of calcium and cysteine and that, under these conditions, the cysteine proteinases were the predominating activities, although the serine and metalloproteinases were also present at lower levels.

This research was conducted to study the hydrolysis of the major oat storage proteins (globulins) by the proteases extracted from germinated oat seeds. Two different analysis methods were employed. In the first, the globulin substrates were incorporated into electrophoresis gels in which the proteinases were separated, which allowed us to study the activities of individual proteolytic enzymes. In the second part of the study the globulins were hydrolyzed in vitro with the proteinases, after which SDS-PAGE analysis was used to analyze the products that were released.

MATERIALS AND METHODS

Seed Germination

Hand-hulled seeds of oat *Avena sativa* L. Veli were surface-sterilized and aseptically germinated as described earlier (Mikola and Mikola 1980) with minor modifications. The seeds were

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treated with 1% sodium hypochlorite for 20 min, washed once with sterile water (5 min), once with 10 mM HCl (10 min), and eight times (5 min each) with sterile water. The surface sterilized seeds were germinated at 16°C on 0.5% sterile agar for eight days. Seed samples (a minimum of 10 seeds) were removed every 24 hr for analysis.

Isolation of Oat Globulins

The oat globulins were isolated from ground oat groats using a modification of the sequential extraction method of Brinegar and Peterson (1982). The albumins were extracted from 1.6 g of meal for 45 min at room temperature with 40 mL of 50 mM tris-HCl buffer, pH 8.0. After centrifugation (10,000 × *g*, 25 min), the globulins were removed from the residue by extraction with the same buffer, to which 1M NaCl was added. After centrifugation, the supernatant was removed and dialyzed overnight at 4°C against two changes (100 volumes each) of deionized, distilled water. The precipitated globulins were collected by centrifugation (10,000 × *g*, 4°C), dissolved in 4 mL of the extraction buffer at 40°C, and used for the analyses.

Enzyme Extraction

Frozen germinated oat samples (minimum of 10 seeds) were homogenized in a 10-mL test tube with a glass rod, or resting seeds were milled in a laboratory mill before extraction. The homogenized or milled seeds were extracted with 1.5 volumes (v/w) of 50 mM sodium acetate buffer, pH 5.0, that contained 0.1 mM ethylenediaminetetraacetic acid (EDTA), and 2 mM cysteine. The extraction was done on a shaker, in ice, for 1 hr. The resulting slurry was centrifuged (10,000 × *g*, 15 min) at 4°C and the supernatant was frozen in aliquots at -20°C until used.

Electrophoresis and the Detection of Endoproteolytic Activities in Gels

The PAGE method and the detection of proteinases after electrophoresis were as described earlier (Zhang and Jones 1995) with modifications. The equipment used was a BioRad MiniProtean system with 1.5-mm thick gels that contained 10 wells. After extraction, the proteinases were separated in a nonreducing 11% acrylamide gel that contained 0.015% (w/v) immobilized oat globulin preparation. After PAGE, the gels were incubated overnight at 40°C in the appropriate buffer. The buffers that were used for incubating the gels were pH 3.8, 200 mM sodium acetate; pH 6.2, 200 mM sodium citrate.

After the gels were incubated to allow the separated proteinases to hydrolyze the incorporated substrate proteins, they were stained with amido black. The activities showed as clear spots on a dark blue stained protein background.

In Vitro Hydrolysis of Oat Globulins

To study the in vitro hydrolysis of oat globulins, the globulins and proteinases were incubated together at 40°C for various times (from 15 min to 24 hr). A globulin preparation (0.125 mL containing ≈0.025%, w/v, globulin) was mixed with 0.5 mL of buffer and 0.375 mL of the enzyme extract. The buffers used were 200 mM sodium acetate buffer, pH 3.8; 200 mM sodium acetate buffer, pH 5.0; and 200 mM sodium phosphate buffer, pH 6.2. When needed, class-specific inhibitors were added at the same time as the enzyme, and reaction mixture included 2 mM cysteine. The reactions were stopped by adding one volume of nonreducing SDS-PAGE sample buffer prepared according to the manufacturers instructions (Bio-Rad laboratories, Hercules CA), and incubating in boiling water for 3 min. The samples were then analyzed by SDS-PAGE in 12% homogenous acrylamide gels and the separated proteins were stained with 0.06% Coomassie Brilliant Blue R-250 in 6% (w/v) trichloroacetic acid.

Inhibition of Endoproteinases by Class-Specific Proteinase Inhibitors

To characterize the activity classes of the endoproteinases, class-specific proteinase inhibitors were added to the hydrolysis reactions according to the method of Zhang and Jones (1995). The concentrations of the inhibitors during testing were E-64 (cysteine proteinase inhibitor), 10 μM; pepstatin A (aspartic proteinase inhibitor), 20 μM; PMSF (serine proteinase inhibitor), 10 mM and; EDTA (metalloproteinase inhibitor), 5 mM. PMSF, pepstatin A and E-64 were purchased from Sigma Chemical Co. (St. Louis, MO), and all other reagents were reagent grade or better.

RESULTS

Formation of Oat Endoproteinases During Germination

Oat samples were collected throughout an eight-day germination period. The primary root emerged after ≈24 hr of incubation. Extracts were prepared and separated on PAGE gels that contained immobilized oat globulin as proteinase substrate. Duplicate gels were run and developed at pH 6.2 (Fig. 1A) and at pH 3.8 (Fig. 1B). At pH 6.2, no proteolytic activity was detected. At pH

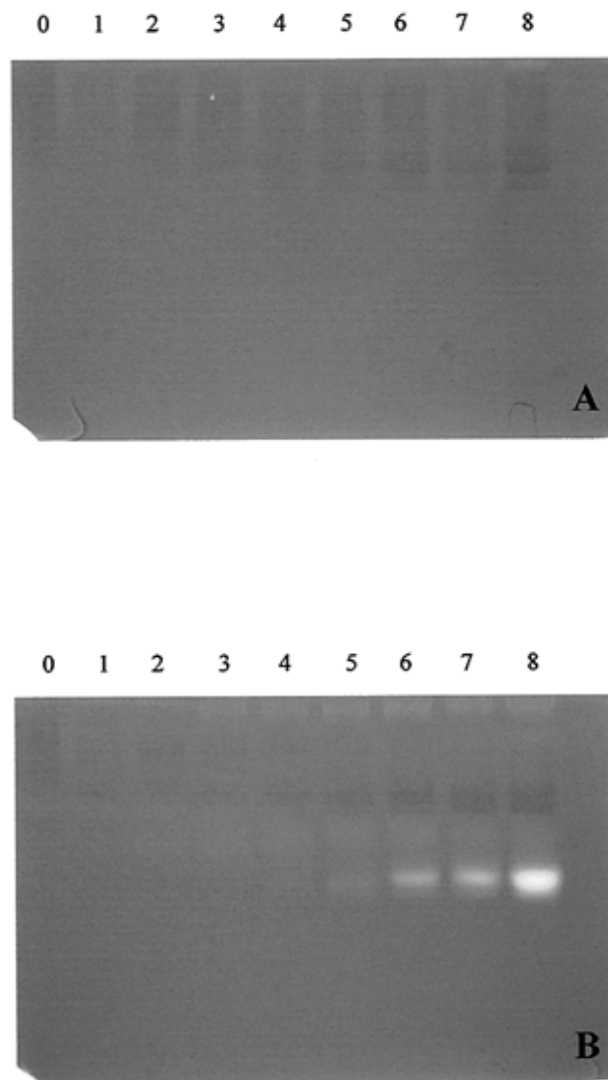


Fig. 1. Changes in oat globulin-hydrolyzing endoproteinase activities during germination. Endoproteinases were extracted from germinated oat samples. Activities were separated by electrophoresis on globulin-containing gels. Proteinases were detected after incubation at pH 6.2 (A) and 3.8 (B). Germination times are indicated above the lanes: 0 = resting seed; 1–8 = seeds germinated for 1–8 days.

3.8, one weak activity was already present in the nongerminated seed, and several weak activities were present after two days (not visible in Fig. 1B). The six- to eight-day germinated samples contained increasing amounts of activity. To ascertain where this activity was situated in the oat kernel, the axis was carefully removed to produce a sample that contained the endosperm (including aleurone) where most of the storage proteins are located. The same activities that are obvious in Fig. 1B were similarly present in the endosperm extract (results not shown).

In Vitro Hydrolysis of Oat Globulins

Extracts of four-day germinated oat endosperms were incubated with a purified globulin preparation for 24 hr at pH 3.8 in the presence and absence of class specific inhibitors (Fig. 2A). SDS-PAGE was used to analyze the hydrolysis products that formed during the hydrolysis. During the incubation in the absence of inhibitors, the nonreduced globulins (indicated by an arrow in Fig. 2A) partially disappeared, and an intermediate molecular weight peptide (IMWP) of MW 34,500 (indicated by an arrow in Fig. 2A) was formed (lane 10 vs. lane 9). The addition of certain class specific inhibitors

altered these results. When E-64 was included in the reaction mixtures it almost totally stopped the hydrolysis (Fig. 2A, lanes 1, 2) whereas the addition of PMSF (lanes 3, 4), pepstatin A (lanes 5, 6) and EDTA (lanes 7, 8) resulted in little or no lowering of the extent of the hydrolysis, compared with the controls (lanes 9, 10). The IMWP was present in all of the reaction mixtures that contained inhibitors other than E-64. Similar experiments also contained mixtures of three of the four inhibitors (Fig. 2A, lanes 12–15). No hydrolysis was detected whenever E-64 was present in the reactions, but whenever it was absent, even in the presence of all three of the other inhibitors, the substrate was partially hydrolyzed and the IMWP was evident. When a second aliquot of enzyme extract was added to one of the reactions after 19 hr of incubation, so that it contained two units of enzyme, no further hydrolysis occurred (Fig. 2B, lane 4 vs. Fig. 2B, lane 3). The nonreduced globulin substrate preparation was nearly homogenous (Fig. 2C, lane 1) and when it was incubated at pH 3.8 without addition of enzymes, it did not change (Fig. 2C, lane 2). Upon reduction of the globulin preparation, subunits were present (Fig. 2C, lane 4) and the IMWP migrated to a position between the subunits (Fig. 2C, lane 3).

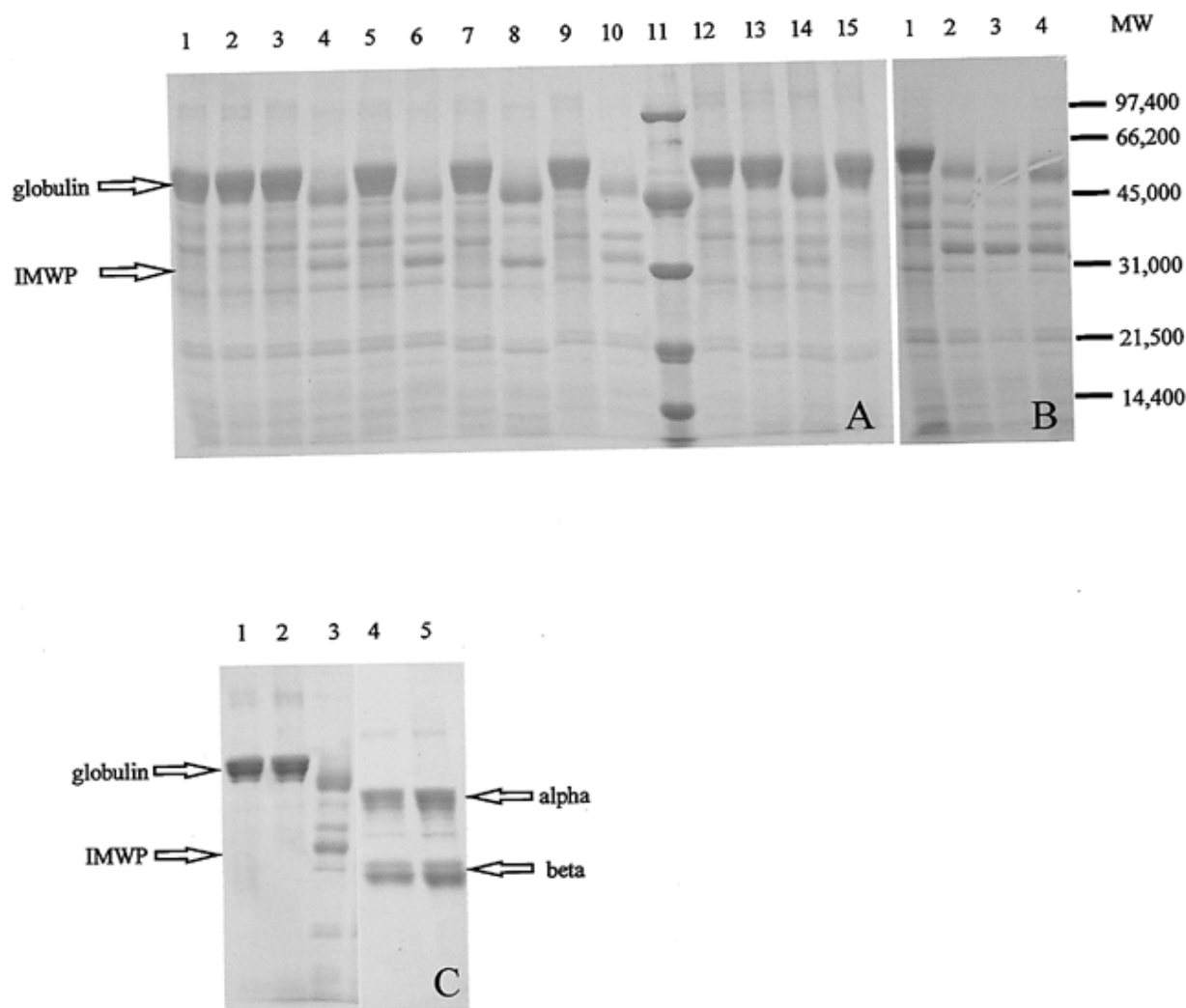


Fig. 2. In vitro oat globulin hydrolysis by endoproteinas from four-day germinated oats. Globulins extracted from resting seeds were incubated at pH 3.8 with endoproteinas extracts. Hydrolysis products were analyzed by SDS-PAGE under nonreducing conditions, except for lanes 4 and 5 in C, in which the samples were reduced with dithiothreitol. Hydrolysis times and inhibitors: **A**, lane 1, 0 hr, E-64; lane 2, 24 hr, E-64; lane 3, 0 hr, PMSF; lane 4, 24 hr, PMSF; lane 5, 0 hr, pepstatin A; lane 6, 24 hr, pepstatin A; lane 7, 0 hr, EDTA; lane 8, 24 hr, EDTA; lane 9, 0 hr, control; lane 10, 24 hr, control; lane 11, molecular weight standard; lane 12, 24 hr, pepstatin A, E-64, PMSF; lane 13, 24 hr, E-64, EDTA, PMSF; lane 14, 24 hr, pepstatin A EDTA, PMSF; lane 15, 24 hr, pepstatin A, E-64, EDTA. **B**, lane 1, as in A lane 9, but with twice as much enzyme, 0 hr; lane 2, as in A, lane 10, but 19 hr; lane 3, as in A, lane 10; lane 4, as in A, lane 10, but a second aliquot of enzyme extract was added after 19 hr of incubation, 24 hr. **C**, lane 1, globulin substrate; lane 2, globulin substrate after 24 hr of incubation; lane 3, globulin and enzyme extract incubated for 1 hr; lane 4, globulin substrate, reduced, 0 hr; lane 5, globulin substrate, reduced, 24 hr. Arrows indicate nonreduced globulins and an intermediate molecular weight peptide (IMWP).

A major globulin hydrolyzing activity appeared on the sixth day of germination (Fig. 1B), but the *in vitro* studies described in Fig. 2 were run using four-day germinated seed extracts, before this activity formed. To study whether the hydrolysis was different with extracts that also contained the major activity, a series of experiments was conducted using eight-day germinated seed endosperm extracts as enzyme sources (Fig. 3). *In vitro* experiments were done with the eight-day extracts to determine the effect of pH on hydrolysis. The globulins and enzymes were incubated together for 24 hr at pH 3.8, 5.0, or 6.2. The globulins were completely hydrolyzed at pH 3.8, although some low molecular weight material was still present (Fig. 3, lanes 1 and 2). At pH 5.0, hydrolysis proceeded at a lower but easily detectable level; the IMWP and some of the native globulins were still present after 24 hr of incubation (Fig. 3, lanes 3–4). Upon hydrolysis at pH 6.2, the intensity of the globulin substrate band was reduced only slightly during the reaction period, but IMWP was formed (Fig. 3, lanes 5–6).

We have previously shown that the addition of cysteine and calcium to reaction mixtures increased the rate at which azogelatin was hydrolyzed by germinated oat endoproteases at pH 6.2 (Mikola and Jones 2000). To determine whether the same effect occurred with the globulin substrate, we tested the effect of these compounds on the hydrolysis of globulins with the eight-day germinated oat endoprotease extract at pH 3.8, 5.0, and 6.2. While the electrophoresis gel results are only semiquantitative, the results (Fig. 3, lanes 7–12) indicated that the addition of 8 mM cysteine and 10 mM calcium did not significantly alter the amount of hydrolysis that occurred at any of the tested pH values.

The effects of class-specific endoprotease inhibitors on the eight-day germinated proteinase activities were analyzed (Fig. 4) at pH 3.8. Because PMSF, pepstatin A, and EDTA were solubilized in methanol, it was added to one of the reaction mixtures (Fig. 4, lane 3) but it did not affect the hydrolysis (Fig. 4, compare lanes 2 and 3). As with the four-day enzyme preparation (Fig. 2A), E-64 almost totally stopped the globulin hydrolysis (Fig. 4, lanes 6–7), and the other inhibitors caused no inhibition (lanes 4–5, 8–11, and 12). Aliquots were removed from the reactions at different times and analyzed (Fig. 5, lanes 1–7). Zero time and 24 hr incubated samples containing E-64 were included (lanes 8–9). After 15 and 30 min (lanes 2 and 3, respectively) of reaction, the IMWP was present but it was greatly diminished in the 45 min

(lane 4) and 1 hr (lane 5) samples and had totally disappeared within 6 hr (lane 6). The addition of E-64 to the reactions almost totally inhibited all hydrolysis. The hydrolysis time experiment was repeated with 8 mM cysteine and 10 mM calcium added to the reaction mixtures, and the addition of these compounds did not affect the hydrolysis rates (results not shown).

There was a protein in the globulin preparation that migrated slightly ahead of the globulin, as a narrow band. It disappeared during the incubations (Figs. 2–5), and this destruction was not prevented by addition of any of the inhibitors. It is not clear whether this band represents a globulin, but it is apparently degraded by a proteinase that is impervious to any of the inhibitors used in this study.

DISCUSSION

We have further characterized the proteinases of germinated oats. Oat globulins, the major oat storage proteins, were incubated with germinated oat endoproteases to see how these poorly studied enzymes affect these “natural” substrates.

The electrophoretically separated enzymes did not hydrolyze the globulins at pH 6.2, the pH of germinating oats endosperm (Fig. 1A), but hydrolysis did occur at pH 3.8, with enzyme preparations prepared from six-day or more germinated seeds. Several weak proteinase activities were also detected as early as the second day of germination but they are not visible in the photographs.

The results obtained using an *in vitro* hydrolysis system generally agreed with those of the gel system. Hydrolysis at pH 3.8 by a four-day germinated endosperm endoprotease extract resulted in partial hydrolysis that yielded an IMWP. This hydrolysis was inhibited by E-64 (Figs. 2A and B).

When eight-day germinated seed endoproteases were studied, the results indicated that total or nearly total hydrolysis of the globulin preparation occurred at pH 3.8 (Figs. 3–5). The IMWP was detected early in the reaction (Fig. 5), but it was then destroyed. As with the four-day germinated enzymes, this eight-day activity was almost totally inhibited by the addition of E-64, a specific inhibitor of cysteine proteinases (Fig. 4). At more neutral pH values (pH 6.2), the hydrolysis was slower and the IMWP was still present after 24 hr of reaction (Fig. 3). The addition of 8 mM cysteine and 10 mM calcium had no effect on the reaction products at any of the pH values tested (Fig. 3).

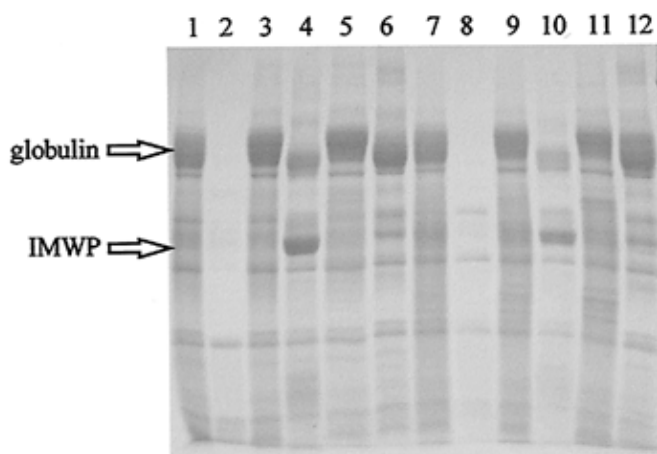


Fig. 3. Effects of pH and addition of calcium and cysteine on *in vitro* hydrolysis of oat globulins by eight-day germinated oat endoproteases. Hydrolysis products were analyzed by SDS-PAGE under nonreducing conditions. Incubations at three different pH levels in the presence of 8 mM cysteine and 10 mM calcium (lanes 7–12) or 2 mM cysteine (lanes 1–6). Lanes 1 and 7, 0 hr, pH 3.8; lanes 2 and 8, 24 hr, pH 3.8; lanes 3 and 9, 0 hr, pH 5.0; lanes 4 and 10, 24 hr, pH 5.0; lanes 5 and 11, 0 hr, pH 6.2; lanes 6 and 12, 24 hr, pH 6.2.

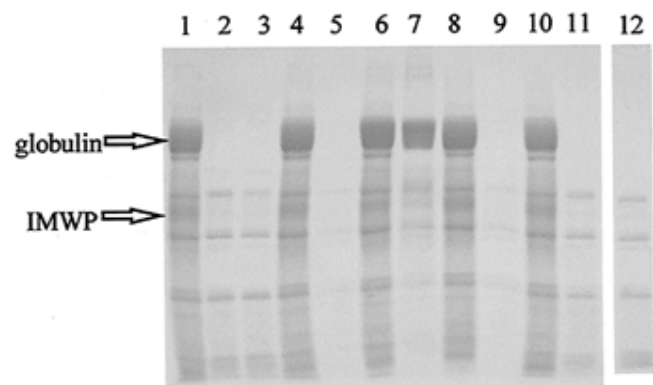


Fig. 4. Effects of class-specific protease inhibitors on hydrolysis of oat globulins by eight-day germinated oat endoproteases. After *in vitro* hydrolysis, hydrolysis products were analyzed by SDS-PAGE under nonreducing conditions. Class-specific inhibitors were added to the reactions as indicated. Lane 1, 0 hr hydrolysis, control; lane 2, 24 hr, control; lane 3, 24 hr, control containing 2% methanol; lane 4, 0 hr, PMSF added; lane 5, 24 hr, PMSF; lane 6, 0 hr, E-64; lane 7, 24 hr, E-64; lane 8, 0 hr, pepstatin A; lane 9, 24 hr, pepstatin A; lane 10, 0 hr, EDTA; lane 11, 24 hr, EDTA; lane 12, 24 hr hydrolysis in the presence of a mixture containing all of the inhibitors except E-64.

Studies of the abilities of cereal endoproteases to hydrolyze globulins are rare. This is probably due to the fact that, in cereals other than oats, the major storage proteins are the prolamins, not globulins. Sarkkinen et al (1992) purified an aspartic proteinase from resting barley seeds and showed that it was unable to hydrolyze endogenous barley embryo or endosperm globulins at pH 4.0. This was apparently not due to the presence of endogenous proteinase inhibitors, because even when the globulin fraction was present, the enzyme still hydrolyzed hemoglobin. Galleschi and Felicioli (1994) have purified an aspartic proteinase from wheat bran and it readily hydrolyzed wheat bran globulins. Finally, this study shows that only the cysteine proteinases of germinating oats can initiate hydrolysis of the oat storage globulins; no evidence was found that aspartic proteinases were involved (Figs. 2 and 4).

At pH 3.8, the cysteine proteinases from four-day germinated seeds initiated globulin hydrolysis, but apparently only gave an incomplete hydrolysis, leaving some of the substrate globulins only partially hydrolyzed (Fig. 2). However at later stages of germination, a globulin hydrolyzing activity appeared (Fig. 1) and cysteine proteinases present in eight-day germinated seeds totally hydrolyzed the globulins (Fig. 4). These results generally agree with the results obtained with barley, a closely related plant, in which it appears that the cysteine proteinases most probably carry out the majority of the hydrolysis of hordeins, the major barley storage proteins. In corn (maize), proteinases that appeared early in germination hydrolyzed the γ -zeins, while different proteinases that occurred during the latter phases of germination preferentially hydrolyzed the α -zeins. In vivo, the γ -zeins also disappeared before the α -zeins (Mitsuhashi and Oaks 1994). Also, in several legumes (review by Shutov and Vaintraub 1987) that contain storage proteins that are related to those of oats, the cysteine proteinases obviously carry out bulk of the hydrolysis. The late appearance of the proteinases that totally hydrolyzed the oat globulins may have been due to the synthesis of new proteinases. Alternatively, the activities of the proteases that were already present may have increased significantly. It is possible that enzymes that were already present, but whose activities were checked by endogenous cysteine proteinase inhibitors, were somehow activated. It is well known that, in barley and barley malt, high levels of protease inhibitors are present that can inhibit the activities of the cysteine class proteinases that are present in germinated barley (Jones and Marinac 1995).

The results of the in vitro analysis of the four-day germinated seed proteinases may partially explain the faint activities that were present at the beginning of germination but were too faint to

reproduce in Fig. 1. Because the proteinases present during the initial phases of germination only partially hydrolyzed the globulins (Fig. 2), some substrate was left in the gel after the incubation was terminated. According to Shutov and Vaintraub (1987), the basic subunits of several leguminous globulins were released from the native protein by a cysteine proteinase and this peptide was then hydrolyzed from the carboxyl terminus by the sequential removal of amino acids. The acid subunits of these leguminous globulins were, in turn, rapidly hydrolyzed to small peptides. In Fig. 3, it appears that the amount of IMWP formed was not proportional to the disappearance of the globulin. The molecular weight of this IMWP, when compared with the molecular weights of the globulin subunits, fell between the molecular weights of the two subunit types (Fig. 4). It is possible that it was derived from the higher molecular weight (α) subunits by a limited hydrolysis. It is also possible that the slightly higher electrophoretic mobility of IMWP, relative to that of the higher molecular weight subunit, was due to intramolecular disulfide bridges that were destroyed when the subunits were analyzed under reducing conditions. However, with the methods used in this study, we could not verify the derivation of IMWP. Determining the amino acid sequence of the IMWP peptide would clarify this point, but was beyond the scope of this study.

This study shows that only the cysteine proteinases hydrolyze oat globulins to any significant extent. Only a little hydrolysis occurred at pH 6.2, which is the pH of the germinating oat endosperm. It is possible that compartmentalization within the endosperm, for example within the protein bodies, could result in a portion of the seed having a pH value low enough to allow these proteinases to operate. The question of whether the pH of the oat protein bodies is different from that of the overall endosperm pH remains to be determined and it is still unknown whether the oat aleurone can acidify its surroundings as barley does (Mikola and Mikola 1980). Detailed studies characterizing the disappearance of the globulins during germination are presently being conducted (T. Sontag-Stroh, *personal communication*), and investigations detailing the occurrence of the IMWP in vivo in the germinating seed also need to be conducted. Our findings that these proteinases are much more active at the lower pH levels strongly implies that these low pH conditions must occur in some areas of the endosperm. It seems unlikely that these proteinases would have evolved under conditions in which they could not operate.

CONCLUSIONS

We have studied the proteinases of germinated oats that hydrolyze the globulins, which are the major oat storage proteins. The hydrolytic activity was very low at pH 6.2, the pH of the germinated oat endosperm. Hydrolysis occurred at an intermediate extent at pH 5.0 and very rapidly at pH 3.8. The major oat globulin hydrolyzing activity appeared after six days of germination. Four-day germinated seed cysteine proteinases hydrolyzed the globulin partially, leaving an intermediate MW peptide of $\approx 34,500$ unhydrolyzed. Eight-day germinated seed cysteine proteinases hydrolyzed the globulins to very small peptides through a 34,500 MW intermediate sized peptide. The cysteine proteinases initiated the hydrolysis. It appears that some pH compartmentalization has to occur within the oat endosperm during germination in order for globulin solubilization to occur.

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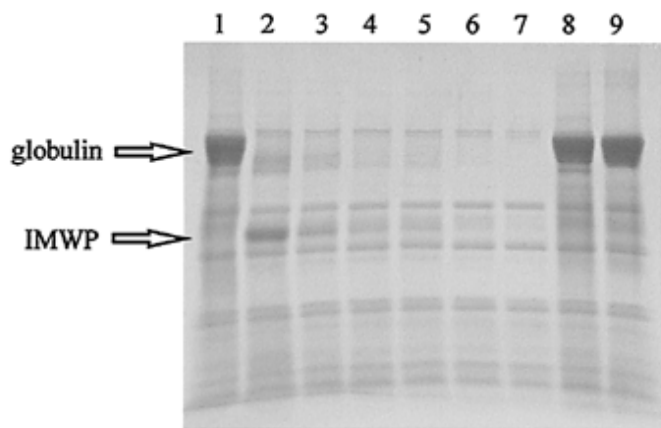


Fig. 5. Time course of in vitro hydrolysis of oat globulin by eight-day germinated oat endoproteases. Reactions at pH 3.8 for different time periods. Hydrolysis products analyzed by SDS-PAGE under nonreducing conditions. Lane 1, 0 min reaction; lane 2, 15 min; lane 3, 30 min; lane 4, 45 min; lane 5, 1 hr; lane 6, 6 hr; lane 7, 24 hr; lane 8, 0 min in the presence of E-64; lane 9, 24 hr in the presence of E-64.

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