

# Characterization of Oat Endoproteinases that Hydrolyze Oat Avenins

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## ABSTRACT

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During oat seed germination, the insoluble storage proteins must be solubilized and transported to the embryo for use by the developing plantlet. We showed earlier that pH 6.2 active serine and metalloproteinases were the predominant gelatin-hydrolyzing enzymes of oats, while the oat globulins were degraded by pH 3.8 active cysteine proteases. The pH of the endosperms of germinating oats is 6.2. We have continued our characterization of the germinated oat proteinases by determining which hydrolyze avenins, the oat storage prolamins. Avenins of resting seeds were purified and hydrolyzed with proteinases that were extracted from oat seeds that were germinated for various periods. The peptides released were analyzed

using SDS-PAGE. The  $\alpha$ -avenins were hydrolyzed at pH 3.8 by cysteine proteinases from four-day germinated seeds and the  $\beta$ -avenins were hydrolyzed by similar enzymes from eight-day germinated seeds. At pH 6.2 or pH 5.0, the avenins were not degraded by any of the germinated oat endoproteinases. It is probable that some kind of pH compartmentalization occurs within germinating oat seed. After four days of germination, either new proteinases form or some preexisting proteinases are activated. The cysteine proteinases are apparently responsible for the majority of the storage protein hydrolysis that occurs during oat germination.

In the resting seed, the storage proteins are generally situated in the endosperm in an insoluble form. During germination, they must be hydrolyzed so that their components can be transported to the embryo for use by the growing plantlet (Bewley and Black 1994). Botanically, germination ends when the rootlet emerges from the grain embryo, but for this report, the term is used in a broader sense, as it is in the malting industry, to include the early phases of plantlet growth (Bewley and Black 1994).

The major storage proteins of oats are globulins (salt-soluble proteins). Avenins, the oat prolamins, are present, but in smaller quantities than the globulins (Peterson and Brinegar 1986). The alcohol-soluble avenins have  $M_r$  22–33 kDa. The avenins of Finnish oat cultivars were investigated by Jussila et al (1992), who detected both  $\alpha$ - and  $\beta$ -avenins in all of the cultivars that they analyzed. The oat globulins and avenins are both present in the protein bodies of the starchy endosperms and aleurone layers of the mature seeds (Donhowe and Peterson 1983).

The proteolytic system of germinated oats has not been well defined. Sutcliffe and Baset (1973) showed that, during germination, the casein hydrolyzing activity that occurred at pH 8.0 increased throughout the entire seven-day germination period, but that after the first two days, the rate of the increase declined. We showed earlier (Mikola and Jones 2000a) that serine and metalloproteinases are the predominant gelatin-hydrolyzing enzymes of germinated oats at pH 6.2, which is the pH of the germinated oat endosperm tissue. It was also evident that the addition of the reducing agent 8 mM cysteine raised the azogelatinase activity by 2.5-fold when 10 mM calcium was also present. Under these conditions (8 mM cysteine, 10 mM  $\text{Ca}^{2+}$ ), the cysteine proteinases were the predominant activities (Mikola and Jones 2000a).

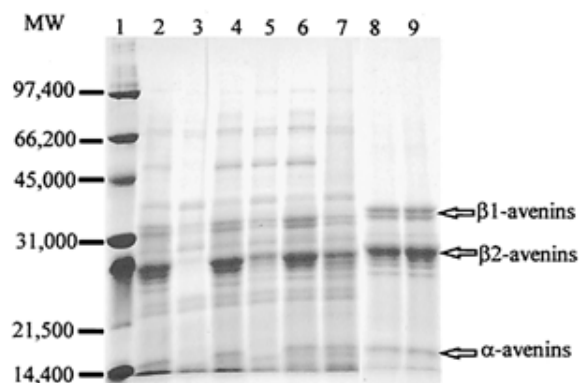
The oat globulins were hydrolyzed at pH 3.8 by endoproteinases that were extracted from germinated oats (Mikola and Jones 2000b). The globulins were partially hydrolyzed by endoproteinases from four-day germinated oats and were completely hydrolyzed by proteinases that were extracted from eight-day germinated seeds. All of these globulin-degrading proteinases were cysteine-class endoproteinases (Mikola and Jones 2000b).

The aim of this study was to continue our characterization of the oat proteolytic system by investigating the germinated oat enzymes that hydrolyze avenins, the oat prolamins.

## MATERIALS AND METHODS

Hand-hulled seeds of the Finnish oat cultivar Veli were surface-sterilized with 1% sodium hypochlorite for 20 min, followed by one wash each with sterile water (5 min) and 10 mM HCl (10 min) and eight washes (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 (Mikola and Jones 2000a). The seed embryos, including the plantlet, were excised and the rest of the seeds were frozen until analyzed.

Avenins were extracted from untreated commercial whole oat meal. The lipids were removed by extracting the meal with acetone (1 g/10 mL) for 30 min with vigorous shaking in a laboratory shaker at 20°C and centrifugation (10,000  $\times$  g, 20°C, 30 min). The albumins were extracted from the resulting pellet with deionized water (10 mL, 20°C) by shaking for 1 hr in a laboratory shaker. After the water-insoluble proteins were removed by centrifugation (10,000  $\times$  g, 20°C, 15 min), the prolamin fraction was extracted from the pellet with 52% aqueous ethanol using the same method that was used to extract the albumins. Pellets were not dried between the extractions. The supernatant from the prolamin extraction was vacuum-dried and suspended in 0.68 mL of 52% aqueous ethanol.



**Fig. 1.** Effect of pH level on in vitro hydrolysis of oat avenins by endoproteinases of eight-day germinated oats. Avenins were extracted from resting seeds and incubated for 0 or 24 hr at pH 3.8, 5.0, and 6.2 with endoproteinase extracts; hydrolysis products were analyzed by SDS-PAGE (12% homogenous acrylamide gels) under nonreducing conditions. Lane 1: molecular weight standards; lanes 2 and 3: pH 3.8, hydrolysis for 0 and 24 hr; lanes 4 and 5: pH 5.0, hydrolysis for 0 and 24 hr; lanes 6 and 7: pH 6.2, hydrolysis, 0 and 24 hr; lanes 8 and 9: avenin substrate preparation incubated in the absence of endoproteinase extract for 0 and 24 hr. Molecular weights of standards (left); areas to which the different avenin groups migrated (right).

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This prolamin fraction was used as a substrate for the endoproteases.

The germinated frozen seeds from which the embryos were removed were homogenized in microfuge tubes using a glass rod, except for the whole resting seeds, which were milled in a laboratory mill (Tecator mill, 0.5-mm sieve). Enzyme extracts were prepared from the homogenized seeds according to the method of Zhang and Jones (1995). The seed homogenates were extracted with 1.5 mL of buffer (pH 5.0, 50 mM Na-acetate buffer that contained 2 mM cysteine)/g of fresh weight, while the ground resting seeds were extracted with the same buffer using a ratio of 4 mL buffer/g of fresh weight. The amount of nitrogen in the avenin preparation was determined using the standard Kjeldahl method and the protein level was calculated ( $N \times 5.7$ ).

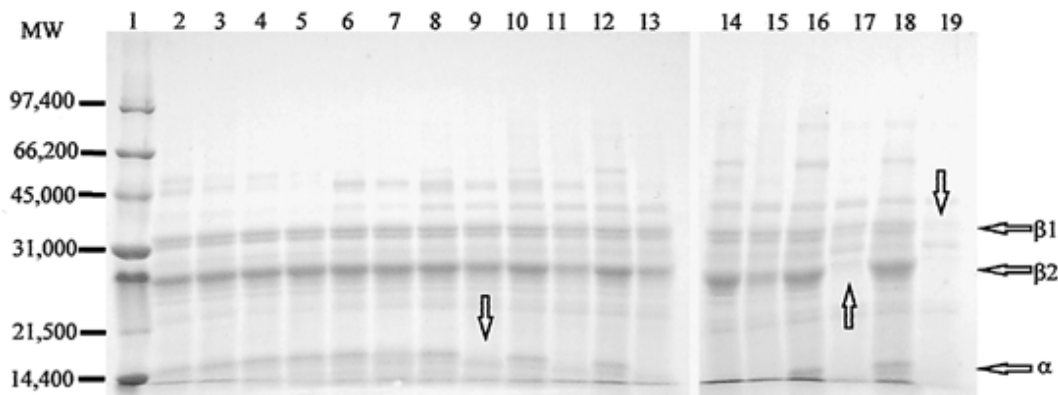
To study the *in vitro* hydrolysis of avenins by the oat endoproteases, the substrate and enzyme proteins were incubated in test tubes at 40°C for various times. The avenin preparation (0.125 mL, containing 1.54 mg of protein) was mixed with 0.5 mL of buffer. The enzyme extract (0.375 mL) was added to this mixture simultaneously with any necessary additives (class-specific inhibitors, calcium or cysteine). The buffers used were Na-acetate for pH 3.8 and pH 5.0, and Na-citrate for pH 6.2. All buffers were 200 mM, and cysteine (2 mM final concentration) was included in each reaction mixture. The reactions were stopped by adding an equal amount of SDS-PAGE sample buffer, which was prepared by mixing 1.0 mL of 0.5M Tris-HCl, pH 6.8, 0.8 mL of glycerol and 1.6 mL

10% SDS, and a trace of bromophenol blue. Immediately after the addition of sample buffer, the sample was incubated in a boiling water bath for 5 min, and the hydrolysis products were separated by SDS-PAGE on 12% homogenous or 8–16% gradient acrylamide gels under nonreducing conditions according to manufacturer's instructions (BioRad, Hercules, CA). Sample (10  $\mu$ L) was applied to each of the 15 electrophoresis unit sample wells. The electrophoresed gels were stained overnight with 0.06% Coomassie Brilliant Blue R-250 in 6% trichloroacetic acid, destained with deionized water until the background was clear, and photographed.

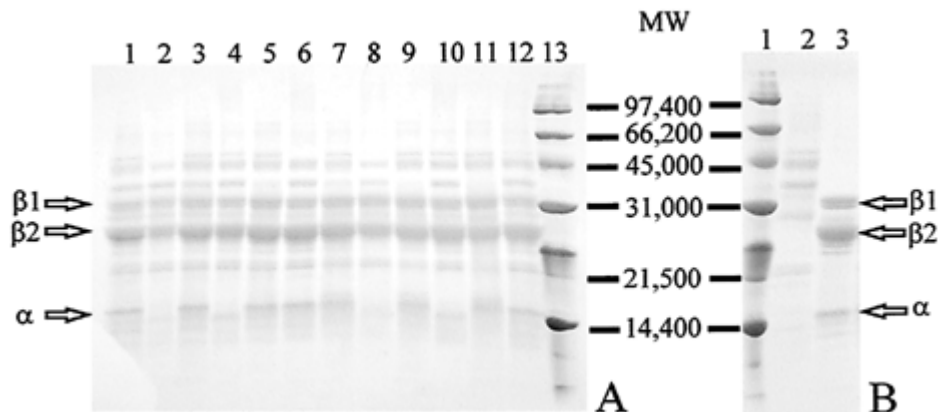
To characterize the endoprotease activity classes, class-specific proteinase inhibitors were added to the reaction mixtures. The final concentrations of the inhibitors tested were 10  $\mu$ M E-64 (cysteine proteinase inhibitor); 20  $\mu$ M of pepstatin A (aspartic proteinase inhibitor); 10 mM PMSF (serine proteinase inhibitor); 1 mM *o*-phenanthroline (metalloproteinase inhibitor), and 5 mM EDTA (metalloproteinase inhibitor). EDTA and E-64 were solubilized in water and the other inhibitors in methanol.

## RESULTS

Seed samples were collected every 24 hr for up to eight days of germination. Enzyme extracts prepared from an eight-day germinated sample were used to conduct preliminary experiments to determine the best pH level for studying the avenin hydrolyzing proteinases. Because the avenins are situated in the endosperm and



**Fig. 2.** Appearance of avenin hydrolyzing proteinases in oats during germination. Proteinase extracts from seeds germinated for different periods, incubated at pH 3.8 with an avenin substrate preparation. Hydrolysis reaction products were analyzed on 12% SDS-PAGE gels. Lane 1: molecular weight standards; lanes 2–19: loaded with reaction mixtures hydrolyzed with enzyme extracts from 0, 1, 2, 3, 4, 5, 6, 7, and 8-day germinated oats, respectively. Even numbered lanes: hydrolyzed for 0 hr; odd numbered lanes: hydrolyzed for 24 hr. Arrows to right indicate  $\alpha$ -,  $\beta$ 1-, and  $\beta$ 2-avenin polypeptide. Arrows in lanes 9, 17, and 19 indicate first hydrolysis of  $\alpha$ -avenins,  $\beta$ 2-avenins and  $\beta$ 1-avenins, respectively.



**Fig. 3.** Effects of pH level and addition of calcium and cysteine on *in vitro* hydrolysis of oat avenins by four-day germinated oat endoproteases. Hydrolysis reaction products were analyzed with 8–16% acrylamide SDS-PAGE gradient gels under nonreducing conditions. **A**, pH 3.8, 5.0, and 6.2 with 2 mM cysteine (lanes 1–6) or 8 mM cysteine and 10 mM calcium (lanes 7–12). Lanes 1 and 7: 0 hr hydrolysis, 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; lane 13: molecular weight standards. **B**, Lane 1, molecular weight markers; lane 2, proteins (enzyme preparation) extracted from four-day germinated seeds; lane 3, avenin (substrate) preparation extracted from resting seeds. Arrows indicate the positions of the  $\beta$ 1-,  $\beta$ 2-, and  $\alpha$ -avenin bands.

they have to be solubilized there, the embryo and plantlet were carefully removed from the seeds before the remaining endosperm material was used for preparing enzyme extracts. The avenin substrate prepared from whole resting seeds (Fig. 1, lane 8) contained both  $\alpha$ - and  $\beta$ -avenins (arrows, Fig. 1). The  $\alpha$ -avenins migrated just behind the ion front, nearly to the bottom of the gel. The  $\beta$ -avenins separated into two fractions, the  $\beta$ 1-avenins, which gave two distinct bands that migrated to a position near that of the 31,000 MW marker protein and the  $\beta$ 2-avenins, whose major bands migrated slightly faster than the 31,000 MW marker protein.

Endoproteinases that were extracted from eight-day germinated oats were incubated for 0 hr (no hydrolysis control) and 24 hr with the avenin preparation at pH 6.2, 5.0, and 3.8 (Fig. 1). A comparison of lanes 8 and 9 shows that no avenin hydrolysis occurred in 24 hr in the absence of added enzyme preparation. The hydrolytic activity was very low at pH 6.2 (lanes 6 and 7) and moderate at pH 5.0 (lanes 4 and 5), while almost complete substrate hydrolysis occurred at pH 3.8 (lanes 2 and 3).

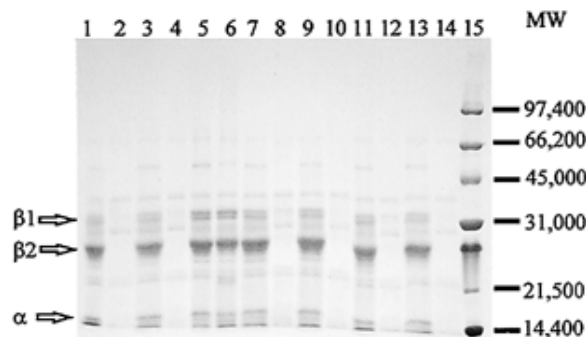
The activities of proteinases that were extracted from seeds germinated for up to eight days were then measured at pH 3.8 (Fig. 2). None of the avenins were hydrolyzed by the enzymes that were extracted from seeds that were germinated for less than three days (lanes 2–7). The  $\alpha$ -avenins (arrow, lane 9) were hydrolyzed by the endoproteinases that were extracted from seeds germinated for three days or longer (Fig. 2, lanes 8–19). The  $\beta$ 2-avenins were partially hydrolyzed by proteinases extracted from the five-day germinated sample (lane 11) and almost totally degraded (arrow, lane 19) by activities extracted from seven-day germinated seeds and almost totally hydrolyzed by eight-day germinated seed enzymes (Fig. 2, lanes 16–19).

Because only the  $\alpha$ -avenins were hydrolyzed at pH 3.8 by the endoproteinases from seeds that were germinated for less than four days, a more careful study of the endoproteinases extracted from four-day germinated seeds was conducted (Fig. 3). The hydrolysis products of these experiments were analyzed with 8–16% acrylamide gradient gels, because the  $\alpha$ -avenins separated better on these than they did on the standard 12% homogenous acrylamide gels (Fig. 3B, lane 3). The protein pattern of the four-day germinated enzyme extract was also analyzed to make it easier to compare which proteins present in the reactions originated from the enzyme extract (Fig. 3B, lane 2) and which from the substrate (Fig. 3B, lane 3). The hydrolysis of  $\alpha$ -avenins (bottom arrow, Fig. 3A) by the four-day germinated seed proteinases was very similar to that catalyzed by the enzymes from eight-day germinated seeds, hydrolysis occurring at pH 3.8 and 5.0, but not at pH 6.2 (compare Fig. 1 with lanes 1–6 of Fig. 3). Because we have shown earlier that addition of 8 mM cysteine and 10 mM calcium raised the azogelatinase activity of oat endoproteinases 2.5-fold (Mikola and Jones 2000a), these compounds were added in the incubation mixtures, but this did not alter the rate of avenin hydrolysis at these pH levels (Fig. 3A, lanes 7–12 vs. lanes 1–6).

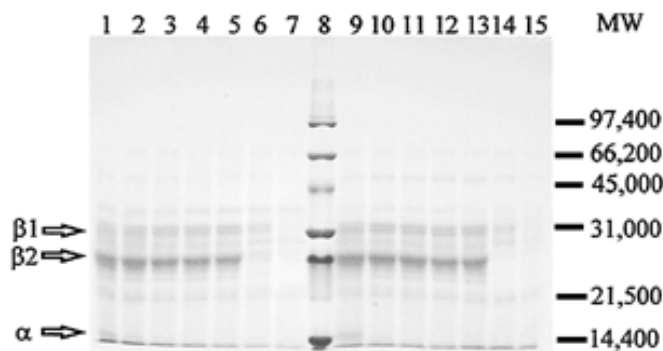
The effects of class-specific proteinase inhibitors on the four-day germinated, pH 3.8-active enzymes were studied (Fig. 4). In the absence of inhibitors (Fig. 4, lanes 1–2), the  $\alpha$ -avenins were readily hydrolyzed, but the addition of E-64, a specific inhibitor of cysteine proteinases, totally inhibited this hydrolysis (Fig. 4, lanes 5–6). There was no apparent inhibition when PMSF (serine proteinase inhibitor, lanes 9–10), pepstatin A (aspartic proteinase inhibitor, lanes 3–4), or *o*-phenanthroline (lanes 7–8) or EDTA (lanes 11–12), which are both metalloproteinase inhibitors, were added to the reactions. Because the PMSF, *o*-phenanthroline and pepstatin A were all dissolved in methanol, a control reaction that contained methanol was included (lanes 13–14). It is apparent that the presence of this solvent did not affect the rate of hydrolysis.

The effects of the class-specific proteinase inhibitors on the pH 3.8 active proteinases that were extracted from eight-day germinated

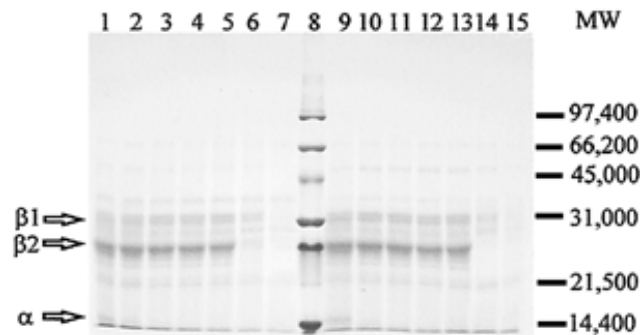
seeds were analyzed using 12% homogenous gels because these gave a better separation of the  $\beta$ -avenins than the 8–16% gels (Fig. 5). As with the four-day germinated grain enzymes, no inhibition of hydrolysis occurred in the presence of inhibitors other than E-64 (Fig. 5, lanes 5–6). The addition of E-64 (Fig. 5, lane 6)



**Fig. 4.** Effects of class-specific protease inhibitors on hydrolysis of oat avenins by four-day germinated oat endoproteinases. In vitro hydrolyses at pH 3.8; hydrolysis products were analyzed on 8–16% acrylamide gradient SDS-PAGE gels under nonreducing conditions. Class-specific inhibitors were added to the reactions as indicated. Odd numbered lanes are control samples that were hydrolyzed for 0 hr, even numbered lanes are samples hydrolyzed for 24 hr. Lanes 1 and 2: enzyme controls with no added inhibitors; lanes 3 and 4: pepstatin A added to reactions; lanes 5 and 6: E-64 added; lanes 7 and 8: *o*-phenanthroline added; lanes 9 and 10: PMSF added; lanes 11 and 12: EDTA added; lane 13 and 14: control with 2% methanol; lane 15: molecular weight standards.



**Fig. 5.** Effects of class-specific protease inhibitors on hydrolysis of oat avenins by eight-day germinated oat endoproteinases. In vitro hydrolyses at pH 3.8; hydrolysis products were analyzed on 12% homogenous acrylamide SDS-PAGE gels under nonreducing conditions. Class-specific inhibitors were added to reactions and reaction times were as in Fig. 4.



**Fig. 6.** Time course of in vitro hydrolysis of oat avenins by endoproteinases extracted from eight-day germinated oats. Reactions at pH 3.8 for different periods of time with 2 mM cysteine (lanes 1–7) or 8 mM cysteine and 10 mM calcium (lanes 9–15). Hydrolysis products were analyzed on 12% homogenous SDS-PAGE gels under nonreducing conditions. Lanes 1 and 9: 0 min; lanes 2 and 10: 15 min; lanes 3 and 11: 30 min; lanes 4 and 12: 45 min; lanes 5 and 13: 1 hr; lanes 6 and 14: 6 hr; lanes 7 and 15: 24 hr; lane 8: molecular weight standards.

resulted in a complete inhibition of the otherwise high (Fig. 5, lane 2) proteolytic activity, indicating that it was the result of cysteine-class proteinases.

Because the  $\alpha$ - and  $\beta$ -avenins were completely hydrolyzed in 24 hr under the conditions used for the uninhibited experiments of Fig. 5, further studies were conducted with shorter reaction periods to determine how the hydrolyses proceeded (Fig. 6). As in the Fig. 5 experiments, hydrolyses were conducted at pH 3.8 using a proteinase extract prepared from eight-day germinated seeds. Under these conditions, the  $\alpha$ -avenin protein bands disappeared within 15 min (Fig. 6, lane 2). The  $\beta$ 2-avenins were almost totally hydrolyzed within 6 hr (lane 6), whereas  $\beta$ 1-avenin bands were still clearly visible after 6 hr of incubation but were hydrolyzed within 24 hr (lane 7). The addition of cysteine and calcium to the reaction mixtures did not affect the hydrolysis rate (lanes 9–15).

## DISCUSSION

The endoproteinases that were extracted from eight-day germinated oat seeds and which hydrolyzed avenins were active at pH 3.8 and completely hydrolyzed the substrate within 24 hr. Proteolytic activities that hydrolyzed the  $\alpha$ -avenins were detected during the early stages of germination, which was three days after imbibition. In contrast, the  $\beta$ 2-avenins were only hydrolyzed by extracts from seeds that had undergone five or more days of germination, and the  $\beta$ 1-avenins were only totally hydrolyzed by enzymes from eight-day germinated seeds. All of these hydrolyses were inhibited by the addition of E-64, indicating that they were catalyzed by cysteine-class proteinases.

Studies on germinated barley endoproteinases have shown that at least two purified cysteine proteinases can hydrolyze hordeins in vitro (Pouille and Jones 1988, Koehler and Ho 1990). Several researchers (Marchylo et al 1986, Pouille and Jones 1988, Weiss et al 1992) have shown that the D hordeins were degraded more rapidly during malting than the B or C hordeins. When a hordein preparation was hydrolyzed in vitro with a cysteine-class endoproteinase that was purified from green malt, the D hordeins were also degraded very quickly (Pouille and Jones 1988). Because the in vivo studies were all conducted with malt, which contains many proteases, there was no way to know whether the D-hordein hydrolyzing proteases formed before those that hydrolyzed the other hordeins, or whether the D hordein protein structure was simply more susceptible than B and C hordeins to the proteinases that were present. In the experiments reported by Pouille and Jones (1988), however, only a single enzyme was present. This protease hydrolyzed all of the proteins present in a hordein extract, but the D hordeins were degraded first, indicating that, in this case, they were more susceptible to hydrolysis than the B and C hordeins.

In studies on the maize (corn) prolamins, the  $\gamma$ -zeins disappeared within the first two days of germination, with the  $\beta$ - and  $\kappa$ -zeins following soon thereafter. The predominant  $\alpha$ -zeins were more stable and persisted until after the other zeins were degraded (Mitsuhashi and Oaks 1994). Mitsuhashi and Oaks (1994) also showed that the cysteine endoproteinases, which appeared early after imbibition, hydrolyzed  $\gamma$ -zeins but not  $\alpha$ -zeins and that the proteinases that appeared later preferentially hydrolyzed the  $\alpha$ -zeins.

We have previously characterized the oat proteinases that hydrolyzed oat globulins (Mikola and Jones 2000b). Proteinases extracted from four-day germinated seeds partially hydrolyzed the globulins, while those from eight-day germinated seeds hydrolyzed the globulins into peptides that were too small to be detected by SDS PAGE on 12% acrylamide gels. These results agreed well with the results obtained in this study, which indicate that a portion of the enzymes that hydrolyzed the oat storage proteins appeared rather

late in the germination process. Other researchers working with corn (Mitsuhashi and Oaks 1994) and barley (Weiss et al 1992) have reported that similar enzyme patterns may also occur in these species. It appears likely that this phenomenon, whereby the various proteases that hydrolyze storage proteins become active at different germination times, may be common among the cereals.

## CONCLUSIONS

Because good protein hydrolysis occurred in this oat system at pH 3.8, which is much lower than the measured pH of the endosperm of germinated oats (pH 6.2), it seems likely that there are areas in the endosperm where the pH level is considerably lower than pH 6.2, which means that pH compartmentalization occurs during germination. This could occur, for example, within the protein bodies where the avenins are situated.

The  $\alpha$ -avenins were hydrolyzed by proteinases that formed in the seed during the early stages of germination, whereas the  $\beta$ -avenins were only hydrolyzed by enzymes that appeared later. These results indicate that the  $\alpha$ - and  $\beta$ -avenins are probably hydrolyzed by different cysteine-class proteinases formed or released at different stages of the germination and early plantlet growth processes. All of the avenin-hydrolyzing proteinases were cysteine-class enzymes.

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