

Comparison of Endoproteinases of Various Grains

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ABSTRACT

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Two-dimensional isoelectric focusing (IEF) × PAGE gels were used to compare the endoproteolytic (gelatinase) activities of germinated barley with those of bread and durum wheat, rye, triticale, oat, rice, buckwheat, and sorghum. Barley was used as the standard of comparison because its endoproteinase complement has been studied previously in the greatest detail. The characteristics of the grain proteases were appraised from their migration patterns and by how they were affected by pH levels. All of the germinated grains contained multiple enzyme activities and their separation patterns and pH levels were at least similar to those of barley. The proteinases of the bread and durum wheats, rye, oat, and sorghum were

most similar to those of barley, whereas the other grains provided more varied patterns. The rice and buckwheat proteinases developed much more slowly than those of the other grains. The activity patterns of the triticale resembled those of the parents, wheat and rye, but the triticale contained many more activities and higher overall proteolytic activities than any of the other species. These results should be applied to scientific or commercial procedures with caution because grains contain potent endogenous proteinase inhibitors that could inactivate some of these enzymes in various tissues or germination stages.

Because of its use as a raw material for preparing malt for brewing, and the importance of the soluble protein level of worts, the endoproteinases of barley (*Hordeum vulgare* L.) have been studied in some detail (Koehler and Ho 1988; Poulle and Jones 1988; Zhang and Jones 1995a,b; Jones and Budde 2003; Osman 2003). A metalloproteinase from buckwheat (*Fagopyrum esculentum* Moench) has been purified and studied (Voskoboinikova et al 1989) and some of the oat (*Avena sativa* L.) and rye (*Secale cereale* L.) proteinases have been partially characterized, but not purified (Mikola and Jones 2000a,b; Mikola et al 2001; Brijs et al 2002). The endoproteinases of most of the other grains have only been studied cursorily, if at all. The proteinases of bread wheat (*Triticum aestivum* L.) have been studied a little, but probably should be looked at in greater detail because their substrates, the wheat proteins, apparently play a large part in determining their baking qualities (Branlard and Dardevet 1985; Wrigley 2003). This article reports a preliminary study that was aimed at determining how the endoproteinase complements of some selected grains differed using a two-dimensional (2-D) isoelectric focusing (IEF) × electrophoresis (PAGE) method (Zhang and Jones 1995a). The enzymes from germinated barley, wheat, oat, rice (*Oryza sativa* L.), buckwheat, triticale (× *Triticosecale* Wittmack), and rye seed were analyzed. For researchers who are interested in modifying the overall proteolytic activities of germinated grains or their by-products such as malt, it is imperative that they understand the endoproteinases because studies have shown that these, rather than the exopeptidases, are the rate-limiting enzymes that control the rate of protein solubilization in barley (Burger and Schroeder 1976; Sopanen et al 1980) and that they start the protein degradation processes in buckwheat (Dunaevsky and Belozersky 1989a,b), oat (Mikola and Jones 2000a,b), and rye (Brijs et al 2002). In germinated barley, which is the best-studied system, the exoproteinases apparently are present in excess and can quickly reduce the large peptides that are released by the endoproteinases to

amino acids and small peptides (Mikola 1983). Generally, mature grain seeds contain only low levels of endoproteolytic activity, and in barley these ungerminated grain proteases mainly belong to the aspartic class (Wrobel and Jones 1992; Zhang and Jones 1999). When the seeds are germinated, however, the proteinase activities increase greatly after ≈1–2 days of germination and reach a maximum after ≈3 days of germination (Wrobel and Jones 1992; Zhang and Jones 1995b). In malted barley, for instance, over 40 different proteolytic activities are present that can hydrolyze either gelatin or edestin (Zhang and Jones 1995a). Although the majority of these activities were due to cysteine-class proteinases, representatives of all four of the common protease classes were present. Similar results were found when oat was malted (Mikola and Jones 2000b), and it seems likely that this is probably a common phenomenon in the various grains. For these reasons, the various grains analyzed in this study were malted and the proteinase complements of the malts were analyzed.

Because the barley endoproteinases have been studied in detail using the 2-D system (Zhang and Jones 1995a,b) and little is known about the characteristics of the proteinases of the other grains, it will be assumed in this article that the members of the four various endoproteinase classes of the other grains migrate in the same way as those of barley with this system. The 2-D analysis system has been successfully applied to germinated oat (Mikola and Jones 2000) and rye (Brijs et al 2002) endoproteinase extracts and, in both cases, the results were similar to those obtained with the barley enzymes. From those data and from the results of this study, it seems likely that the enzymes composing the different protease classes of the various grains will migrate similarly in the 2-D system. It will remain for other researchers to use the specific endoproteinase inhibitors to prove with certainty that this is or is not the case. Although the methods used do not and cannot completely and definitively define the characteristics of all of the proteinases of these species, the results obtained do give a good idea of the similarities and differences among the enzymes of the various species and will provide a good starting point for researchers who want to conduct further studies.

MATERIALS AND METHODS

Materials

The grains used in this study were kindly supplied by the following researchers: G. Lookhart and D. Wesenberg (wheat); J. Dexter (durum wheat, *Triticum durum* Desf.); L. W. Rooney (sorghum, *Sorghum bicolor* L. Moench and *S. vulgare*); R. Duerst (rye and oat); C. Bergman (rice); D. Wesenberg (triticale); and C. Campbell (buckwheat).

¹ USDA-ARS, Cereal Crops Research Unit, 501 N. Walnut St., Madison, WI 53726. Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

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Malting

The samples were all cleaned, steeped, and germinated for four days, as reported earlier for barley samples (Jones et al 2000). They were not kilned to ensure that no proteolytic activities were destroyed during the heating process. Some of the grains germinated more slowly than barley, in which case they were subjected to extended periods of germination until their rootlets were ≈ 7 mm long.

Enzyme Extraction

The green (unkilned) malts were extracted at a ratio of 1 g of malt to 2 mL of buffer at 4°C using a buffer that contained 100 mM sodium acetate, 2 mM cysteine, and 0.1 mM EDTA, adjusted to pH 4.7. The germinated seed were suspended in the buffer, crushed in a mortar and pestle, and washed into a plastic test tube, where they were subjected to three 1-min cycles of homogenization (Polytron, Brinkmann, Lucern, Switzerland). They then were centrifuged for 20 min at 10,000 rpm at 4°C and the supernatants were removed and dialyzed overnight in 3,500 MW cutoff tubing against 4°C, 5 mM sodium acetate, pH 5.0.

Separation and Detection of Endoproteases

The enzyme preparations were subjected to separation by a 2-D IEF \times PAGE method conducted on acrylamide gels (Zhang and Jones 1995a). The second-dimension (PAGE) gels contained incorporated gelatin. The separated endoproteases were detected by incubating the second-dimension gel in buffers adjusted to pH 4.8, 6.0, or 3.8, as needed. The separated enzymes that were active at the incubation pH hydrolyzed the gelatin that was incorporated into the gel and, when the gel was stained for protein, the areas to which the various endoproteases had migrated did not stain, but showed up as clear spots against a blue background.

For ease of presentation and clarity, drawings of the resulting gels have been made and are shown in the accompanying figures.

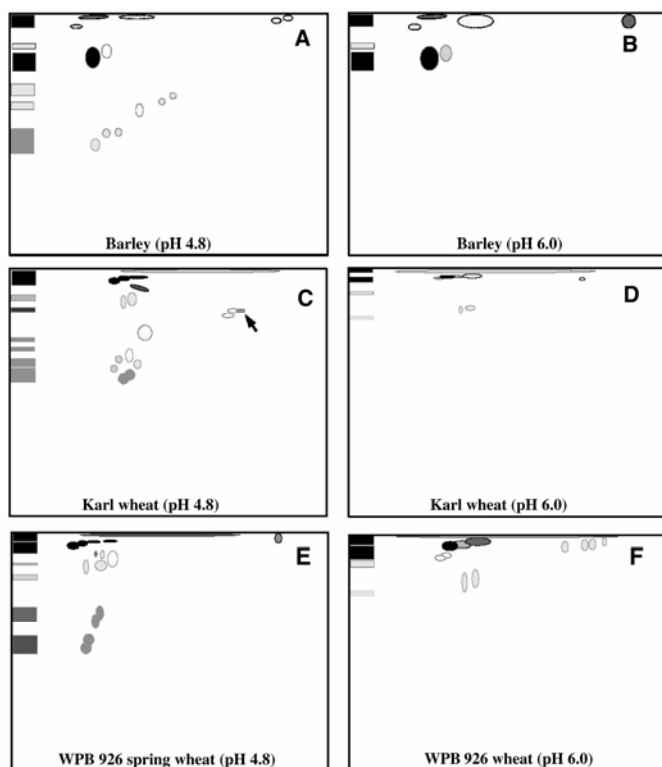


Fig. 1. Diagrams of two-dimensional IEF \times PAGE separations of germinated barley and bread wheat seed. **A** and **B**, barley extracts; **C–F**, bread wheat extracts. Bands on the left side of the gels indicate extract samples applied after IEF separation (thus subjected only to PAGE separation).

These drawings are negatives of the real gels, in that the enzymatic activities are shown as dark spots against a clear background; whereas, in the experimental gels, they show up as clear areas against a blue background. The activities varied greatly, from giving a slight diminution of the blue background color to completely clearing it. This is indicated in the drawings by varying the intensities of the spots from light (little activity) to black (complete clearing of the gel).

As a check of how well the 2-D separations worked and to demonstrate that some information can be obtained by applying only the PAGE separation, samples of the enzyme extracts also were applied to a slot on the left-hand side of the PAGE gel after the IEF separation had been conducted. Thus, they were subjected only to the PAGE separation but their activities were measured together with those of the 2-D separated enzymes. The separations were conducted in duplicate and if the duplicate patterns were not the same, they were rerun until reproducible gels were obtained.

RESULTS AND DISCUSSION

Separation of Endoproteases of Malted Barley

Because the endoproteases of barley grain and barley malt have been studied much more thoroughly than those of any of the other grains, the endoprotease (gelatin-degrading) enzyme patterns of the grains being investigated were compared with those of barley green malt. The 2-D separation patterns of malted barley endoproteases that were active at pH 4.8 and 6.0 are shown in Fig. 1A and B, respectively. The activities of the enzymes at these pH levels were studied because the pH inside a germinating barley grain is apparently pH 4.8 (Cynthia Henson, *personal communication*) and the pH of U.S. brewing mashes averages pH 6.0 (Jones and Budde 2003). Therefore, the endoproteases that are active at these pH levels are the ones that are presumably most important to the seed germination and to the commercial brewing processes.

As shown previously, ungerminated barley contains little endoproteolytic activity or endoproteases (Wrobel and Jones 1992). During the seed germination (or malting) process, the endoproteases form (or, less likely, some inactive proteases are activated) until, after ≈ 3 days of germination, they are present at their maximal levels (Wrobel and Jones 1992; Zhang and Jones 1995b). At this time, as many as 42 different proteolytic activities are detectable in barley malt (Zhang and Jones 1995b). Some of these presumably consist of isoenzyme forms, but this has not yet been established. Among these are four classes of barley endoprotein-

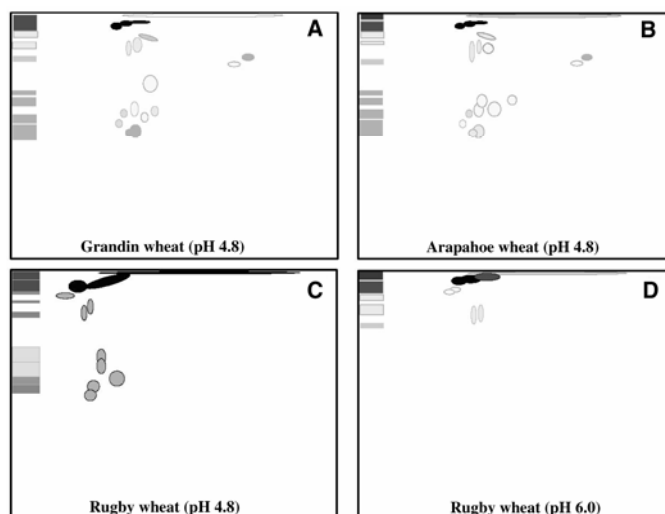


Fig. 2. Diagrams of two-dimensional IEF \times PAGE separations of germinated bread wheat and durum wheat seed. **A** and **B**, bread wheat extracts; **C** and **D**, durum wheat extracts.

ases that can be identified readily by their susceptibilities to chemical inhibitors. The endoproteinasomes composing these four classes tend to migrate to different areas of the 2-D gels, the metallo- and serine proteinases remaining near the top of the gel and the aspartic enzymes migrating further into the gel, with all of the activities that move to the front half of the pattern being due to the various cysteine class proteinases (Zhang and Jones 1995a).

The activities of the various endoproteinasomes of barley are very pH dependent. The metallo- and serine enzymes are active between pH 4.8 and 8.5, whereas the cysteine and aspartic class enzymes are most active between pH \approx 3.0 and 5.0 (Zhang and Jones 1995a). Thus, by running identical gels and staining them at different pH levels, it is possible to readily differentiate the cysteine class endoproteinasomes from those of the other classes.

The proteinase classes of the various activities can be determined by developing the separated activities in the presence of the various chemical inhibitors that are specific for the different classes (E-64 for the cysteine enzymes, pepstatin A for aspartic endoproteinasomes; 1,10-phenanthroline for the metalloproteinases; and phenylmethylsulfonyl fluoride for the serine proteinases) (Zhang and Jones 1995a). But that was not done in this experiment because it was designed to determine whether the proteinase compositions of the various grains and some of their cultivars differed, and not to prove the exact makeup of the compositions of any one species. The aspartic endoproteinasomes of malted barley do not readily hydrolyze the substrate gelatin; therefore, studies of these enzymes generally have been conducted using the substrate edestin, a storage protein from *Cannabis sativa*. Because this protein is no longer readily available, it was not used for this study. From their migration rates, however, it appears that the germinated grains of some of the nonbarley species did contain aspartic class proteinases that were detectable with the gelatin substrate that was used.

Endoproteinasomes of Malted Bread Wheat

Samples of grain of various hexaploid wheat classes were malted and analyzed (Figs. 1 and 2). The proteinase complements of the hard red winter wheat cultivar Karl (Fig. 1C), the soft winter wheat cultivar WPB 926 (Fig. 1E), the hard red spring wheat cultivar Grandin (Fig. 2A), and the winter wheat cultivar Arapahoe (Fig. 2B) that were active at pH 4.8 were all very similar. They apparently contained several highly active metalloproteinases and groups of less active serine and cysteine enzymes. Karl, Arapahoe, and Grandin also contained an activity that migrated strongly in the IEF separation and that moved more slowly during the PAGE separation (indicated by the arrow in Fig. 1C). When the Karl and

WPB 926 gels were developed at pH 6.0 (Fig. 1D and F), the cysteine proteinases were completely inactivated, as were their barley counterparts (Zhang and Jones 1995a). The activity indicated in Fig. 1C also was inactivated, so it was also likely a cysteine enzyme. In contrast to the situation in barley, the wheat serine endoproteinasomes were less active at pH 6.0 than at 4.8.

Durum Wheat Endoproteinasomes

When the endoproteinasomes of the durum wheat cultivar Rugby were extracted, separated, and analyzed (Fig. 2C and D), the results were essentially identical to those obtained with Karl. The metalloproteinase activities of the durum extract may have been slightly more active than those of Karl at both pH 4.8 and 6.0, but this is not certain because the analytical method used was only semiquantitative. It is interesting that the metalloproteinase activities of both the hexaploid and tetraploid malted wheats were apparently very active because little notice has ever been paid to this group of wheat proteinases. We recently showed that a similar situation exists in malted barley where the metalloproteinases are responsible for releasing as much soluble protein during the mashing phase of brewing as the cysteine class enzymes, which previously were thought to be the major protein-hydrolyzing enzymes of malt (Jones 1999; Jones and Budde 2005).

Endoproteinasomes of Rye

Two Midwestern rye cultivars, Spooner and Ryman, were malted and extracted and their endoproteinasomes were analyzed. The enzymes active at pH 4.8 (Fig. 3A and C) were more similar to those of barley than to the wheat enzymes, in that they contained relatively large amounts of the serine enzymes and these maintained their activities at pH 6.0 (Fig. 3B). They also contained multiple activities at pH 4.8 that migrated to the upper right corner of the gels, as did the second group of barley serine proteinases. Also, like the barley enzymes, one of these serine enzymes was inactive at pH 6.0, whereas the other remained active. The rye contained many more cysteine class proteinases than the wheat, and even

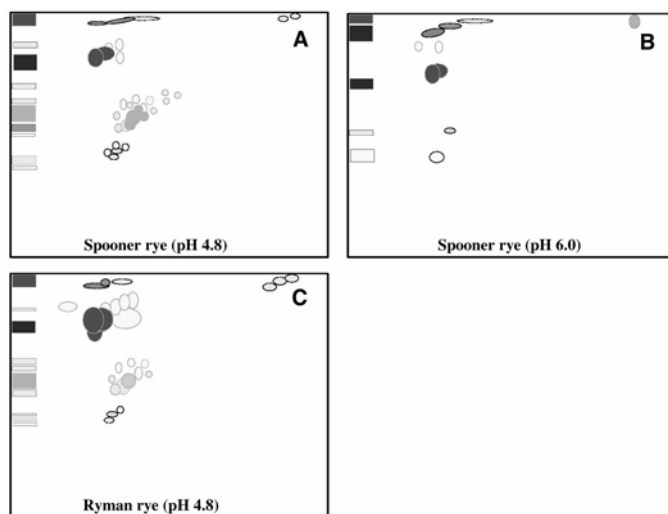


Fig. 3. Diagrams of two-dimensional IEF \times PAGE separations of germinated rye seed.

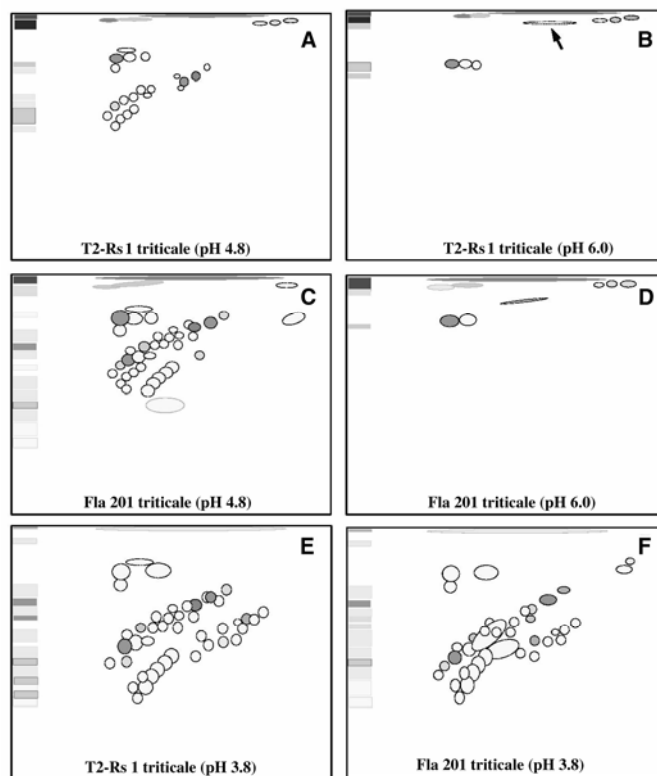


Fig. 4. Diagrams of two-dimensional IEF \times PAGE separations of germinated triticale seed.

more than did barley. The effect of changing the development to pH 6.0 was similar to what was observed with the barley enzymes (Fig. 3B), except that a couple of the cysteine proteinases retained activities at pH 6.0, which none of their barley counterparts did.

Triticale Enzymes

Triticale is a manmade species that combines the genomes of wheat and rye parents. Two triticale lines, T2-Rs 1 and Fla 201, were obtained, malted, and analyzed. The endoproteinase patterns of the two different triticale lines were very similar to each other (Fig. 4A–F), quite different from those of barley (Fig. 1A and B), and showed some similarities of those of wheat and rye. At pH 4.8 (Fig. 4A and C), the metalloproteinase pattern was similar to those of wheat, with a long, smeared, slowly migrating activity that was not present in the rye extracts. The main serine enzyme pattern also was similar to those of wheat, containing several relatively low-activity enzymes, whereas the high pI serine pattern was like that of rye, containing multiple enzymes. Both triticale lines contained many more cysteine-class proteinases than any of the other grains that were analyzed.

To ensure that this was not an artifact, an extra gel containing extracts of each of the triticale lines was run and developed at pH 3.8 (Fig. 4E and F). At this pH level, even more cysteine proteinase activities were present, whereas the high-pI serine activities were inactivated. This is consistent with what was found previously with barley enzymes (Zhang and Jones 1995a). All of these cysteine-class enzymes were inactive in gels developed at pH 6.0 (Fig. 4B and D), indicating that they were indeed cysteine enzymes. Some of the multiple enzymes that were active at pH 3.8 and 4.8 and migrated to the serine location were inactivated at pH 6.0, which may indicate that they were really aspartic-class enzymes that, unlike their barley compatriots, were able to hydrolyze gelatin. There was one enzyme (arrow in Fig. 4B) that occurred in both triticales and was active at pH 6.0 but not at lower pH values. No similar enzymes were ever seen in barley, rye, or wheat, and it would be interesting to know to which class it belongs.

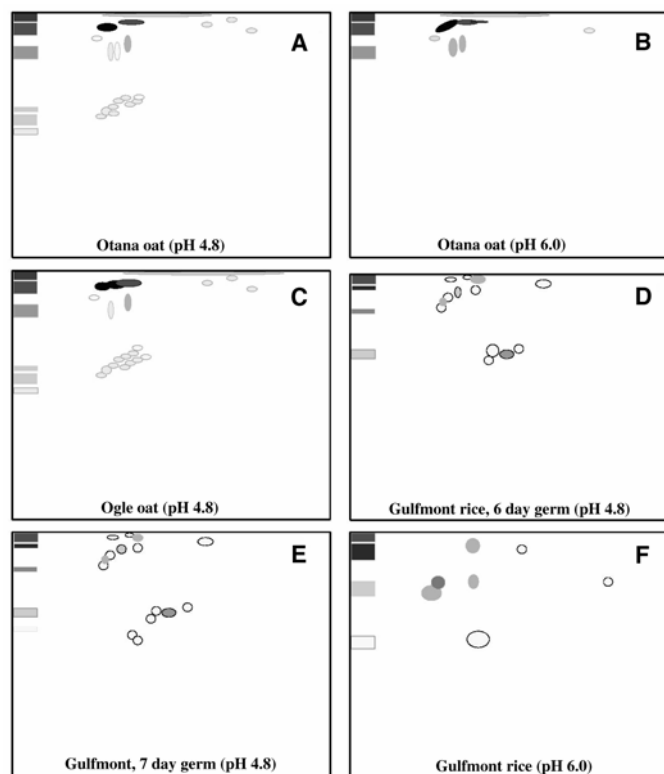


Fig. 5. Diagrams of two-dimensional IEF \times PAGE separations of germinated oat and rice seed. **A–C**, oat extracts; **D–F**, rice extracts.

Probably the most interesting thing about the triticale results was the fact that they contained many more endoproteinases than any of the other grains that were analyzed. It seemed probable that this plethora of proteinases would result in malted triticale with an overall endoproteinase activity greater than those of the other species. To determine whether this was the case, triticale and Harrington barley were grown under dryland and irrigated conditions at two locations in Idaho. After they were malted and mashed, the soluble protein contents of the worts were 4.28% (dryland barley); 6.59% (triticale); 4.31% (irrigated barley); 5.32% (triticale). In terms of the percentages of the total protein contents that were solubilized, the triticale values were 122% (dryland) and 121% (irrigated) of those of barley. In light of these results, it seems that anyone wanting to hydrolyze proteins enzymatically would be well advised to test triticale malt as a potential source of enzymes.

Oat Endoproteinases

Oat lines differ from barley, wheat, and rye in that their major seed storage proteins are globulins rather than prolamins. Because they need to hydrolyze these different proteins during germination, it seemed probable that they might produce a different set of proteinases for this process. However, at pH 4.8, the 2-D PAGE patterns of two cultivars analyzed (Otana and Ogle) were very similar to those of the prolamin-containing species (Fig. 5A and C). Also, like the other species, it was primarily the activities of the cysteine-class enzymes that were inactivated at pH 6.0 (Fig. 5B).

Although these results are not necessarily what one would expect, they agree very well with the results of Mikola and Jones (2000b), who reported that, with the 2-D system and development at pH 6.2, only serine- and metalloproteinases were detected; whereas with a soluble analysis system using the substrate azogelatin, cysteine-class proteolytic activity was present and predominated over the other activities. The cysteine proteinases were able to hydrolyze both oat globulins and avenins, the oat prolamins (Mikola and Jones 2000a; Mikola et al 2001). Mikola concluded that both types of oat storage proteins were hydrolyzed mainly by the cysteine proteinases; therefore it is not so surprising that their endoproteinase complements are quite similar to those of the other species.

Rice Proteinases

Because rice is closely related to wheat and oat, it seemed likely that its malt would contain a complement of endoproteinases similar to theirs. After the normal four days of germination, the rice cultivar Gulfmont contained only small amounts of proteolytic activity (results not shown); therefore, its germination period was extended to six and then seven days. The results are shown in Fig. 5D and E. Even after these extended germination times, the rice proteolytic activities were still relatively low. In addition, the rice did not contain the highly active metalloproteinase enzymes that form the long line across the top of the wheat, oat, triticale, and buckwheat gels. The malted rice did contain several low-activity proteolytic activities that migrated to the metallo- and serine-proteinase area of the gel, and a second group of low-activity enzymes that migrated to the cysteine area. When the gel development was conducted at pH 6.0, some of the slowly migrating activities were increased and most of the cysteine class activities were inactivated. Extending the germination time from six to seven days had little effect on the enzyme composition. These results seem to indicate that the proteolytic hydrolyses that occur in rice during germination may be similar to those that occur in wheat, rye, and oat (via cysteine class proteases), but they probably occur at a slower rate and because the serine- and metalloproteinases are so dissimilar, the overall process may occur very differently.

Buckwheat Proteases

The seed storage proteinases of buckwheat, like those of oat, are composed primarily of globulins; therefore, it seemed probable

CONCLUSIONS

that their endoproteinase complements also might be similar. But this did not prove to be the case. After the normal four-day germination period (Fig. 6A), the prevalent activity present was the metalloproteinase activity that forms the very elongated activity that stays near the top of the PAGE gel. Some spots of very light activity migrated to the serine- and cysteine-proteinase gel locations but these were insignificant compared with the metalloproteinase activity. To increase the amounts of enzymatic activity, maltings were conducted with germination periods of six and seven days (Fig. 6B and C). Increasing the germination times increased the number of activities that were detectable in the serine- and cysteine-enzyme gel areas but these activities were still meager compared with the metalloproteinase activity.

These findings fit well with those of Dunaevsky and Belozersky, who reported that the storage globulins of buckwheat initially were hydrolyzed by a metalloproteinase (Dunaevsky et al 1983) that they have purified and characterized (Voskoboinikova et al 1989). After initial hydrolysis, they found that the globulin breakdown apparently was completed by a cysteine-class proteinase, possibly with the assistance of a carboxypeptidase (Dunaevsky and Belozersky 1989b).

Endoproteinases of Malted Sorghums

When the endoproteinase activities of malted commercial red and white sorghum samples were analyzed at pH 4.8, high levels of metalloproteinases again were present, especially in the white sorghum (Fig. 6D and E). The sorghum also contained what were apparently serine- and cysteine-class proteases. The overall sorghum protease patterns are similar to those of the barley-wheat-rye group, but most of the individual cysteine proteinases had lower pI levels. Nothing has been published previously about the proteinases of sorghum; however, from these data it seems likely that metalloproteinase may play an important role in degrading their seed storage proteins.

This is the first comparison that has been made of the endoproteinases that occur in various germinated grains. It indicates that the endoproteinase complements of barley, wheat, rye, triticale, and oat are basically similar. The sorghum enzyme patterns also showed similarities with the barley enzymes but were clearly different. The rice and buckwheat patterns, however, differed strikingly from those of the barley group and from each other. It seems strange that the buckwheat and oat proteinase complements are so different because both store their seed amino acids as globulin proteins instead of the more common prolamins. They also are similar in that the initial steps of their storage protein degradations are catalyzed by noncysteine proteases (metalloproteinases in buckwheat, serine enzymes in oat), after which the cysteine-class enzymes perform the majority of the remaining protein degradation. The pH 6.0 rice protease pattern was especially different from those of the other grains studied.

One of the most interesting facts resulting from this study is that most of the malted grains contain relatively large amounts of proteinases that migrate like the metalloproteinases of barley. In the past, it was assumed that the metalloproteinases played little or no role in the degradation of storage proteins, except in the case of buckwheat. Recently, however, we have shown that the addition of the metalloproteinase inhibitor 1,10-phenanthroline to barley malt mashes inhibited the release of soluble protein (soluble protein, peptides, and amino acids) into brewing worts as strongly as inhibiting the cysteine class endoproteinases (Jones and Budde 2005). Considering the results from the earlier buckwheat and barley mash studies and from these experiments, it seems likely that the metalloproteinases play a much larger role in storage protein degradation in grains than thought previously. The only two grain metalloproteinases that have been purified and studied to date are those from buckwheat (Voskoboinikova et al 1989) and barley (Fontanini and Jones 2001). One reason for this is that these enzymes are notoriously hard to work with. However, it appears that, if we are going to truly understand the storage protein hydrolysis process in grains, more of these metalloproteinase studies are needed.

Finally, in trying to define the processes whereby grain storage proteins are degraded, researchers must always bear in mind that it is not just the grain and malt endoproteinases that must be investigated. There are a whole series of compounds in dormant and germinated grain that can interact with the proteinases that are present and thereby stop these enzymes from functioning. Endogenous inhibitors have been described that can inactivate the metalloproteinase of buckwheat (Belozersky et al 1982; Elpidina et al 1991) and proteins have been purified and studied that strongly inhibit the cysteine proteinases of barley and some of the serine endoproteinases of barley (Jones and Marinac 1997, 2000; Jones and Fontanini 2003). Undoubtedly, similar or different proteolytic inhibitors occur in the other grains, and these need to be looked for. Just because a particular proteolytic enzyme occurs in a seed does not mean that it will be active there. If it is associated with an inhibitor, it will not be able to cleave any peptide bonds. This situation will be even more complicated if the inhibitors sometimes interact and at other times do not, in order to regulate protein hydrolysis in the various tissues and at the different stages of germination.

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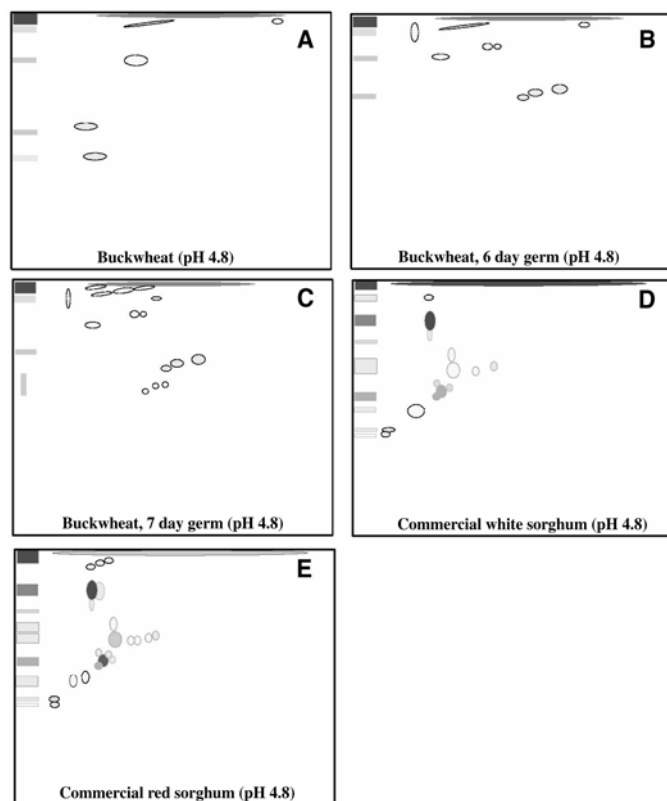


Fig. 6. Diagrams of two-dimensional IEF \times PAGE separations of germinated buckwheat and sorghum seed. **A–C**, buckwheat extracts; **D** and **E**, white and red commercial sorghum extracts.

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