Study of Metallopeptidase Isozymes from Malted Barley (*Hordeum vulgare* cv. Morex)

D. Fontanini*,† and B. L. Jones†,‡

Department of Agronomy, University of Wisconsin—Madison, Madison, Wisconsin 53706, and Cereal Crops Research Unit, Agricultural Research Service, U.S. Department of Agriculture, Madison, Wisconsin 53705

It has been reported that germinated barley contains peptidases that are sensitive to metal-chelating agents; however, none of these enzymes have been isolated, nor have their roles in germinated barley been investigated. Anion-exchange chromatography and chromatofocusing have been used to isolate a group of peptidases from barley (*Hordeum vulgare* cv. Morex) green malt that are sensitive to metal-chelating agents. Their activities were studied using one- and two-dimensional polyacrylamide gel electrophoresis. When analyzed on two-dimensional PAGE gels that contained gelatin as substrate, the enzymes separated into three major and approximately six minor activity spots with acidic p*I* values. The enzymes were optimally active against the gelatin substrate at pH 8.0 and were completely inhibited by 1,10-phenanthroline and EDTA, indicating that they belonged to the metallopeptidase class (EC 3.4.24.*x*). After the enzymes were inhibited with EDTA, the activities were recovered in the presence of low concentrations of metal ions. The hydrolysis of gelatin substrate was also impaired by the presence of reducing agents. The metallopeptidases readily digested, in vitro, the barley prolamine D hordein, indicating that they may be involved in degrading storage proteins during barley germination.

Keywords: *Barley; germination; hordein; Hordeum; malting; metallopeptidase; protease; protein hydrolysis*

INTRODUCTION

It is well recognized that germinating plant seeds contain endopeptidases. Although some of these enzymes are already present in the developing and dry seeds, most are synthesized or activated at the onset of germination (*1*). The main substrate targets of many of the seed peptidases are the storage proteins that have been accumulated in the endosperm during seed maturation. It is through the hydrolysis of these proteins that the embryo, and eventually the seedling, obtain the supply of nitrogen compounds that is necessary for the early stages of plant growth.

The hordeins are prolamines; they occur abundantly and serve as storage proteins in the barley seed. They comprise a complex polymorphic mixture that can be resolved by SDS-PAGE into three groups, the B, C, and D hordeins (*2*). It has been demonstrated that the cysteine class peptidases are the main enzymes responsible for hydrolyzing the bulk of the barley storage proteins (*3*−*6*). Experiments on the in vitro hydrolysis of hordeins by these purified enzymes have shown that they quickly hydrolyze the B, C, and D hordeins (*3*, *5*, *6*).

Barley seeds also contain aspartic peptidases, serine peptidases, and metallopeptidases. The one aspartic peptidase that has been purified from resting barley seeds was closely related to mammalian cathepsin D, and it did not hydrolyze storage proteins (*7*). Nevertheless, a group of aspartic peptidases has been identified in germinating barley seeds that might play a role in mobilizing storage proteins because they hydrolyzed chloroform/methanol extractable barley proteins in vitro (*8*). Proteases that belong to the serine class have been identified in green malt (*9*, *10*), and hordolisin, a serine peptidase, has been purified from barley green malt (*11*). It was homologous to the plant subtilisin-like serine peptidase, cucumisin. The authors excluded that the enzyme might play a role in hydrolyzing storage proteins during barley germination. However, two serine peptidases that were purified from germinating seeds of the dicotyledonous plants *Vigna mungo* (*12*) and soybean (*13*) both partially hydrolyzed globulin storage proteins. It was postulated that they may play a physiological role in degrading storage proteins during germination.

Barley endopeptidases that were inhibited by EDTA were first identified by Enari and Mikola (*14*) in green malt. A partial characterization of the neutral peptidases from 4-day-germinated barley seed extracts by Wrobel and Jones revealed the presence of five metallopeptidases that had molecular weights >164 kDs (*15*). These enzymes were inhibited by metal-chelating agents, and their activities were restored by metal ions. Metallopeptidases, especially the matrix metallopeptidases (MMPs) of animal systems, have generated considerable interest because of their wide distribution in extracellular matrices and their regulatory functions. The roles of such enzymes in plants, and in particular in cereal seeds, have not been clarified yet. One metallopeptidase from resting buckwheat seeds has been purified and characterized (*16*). It hydrolyzed the 13 S buckwheat

* Address correspondence to this author at 501 Walnut St., Madison, WI 53705-2334 [telephone (608) 262-4480; fax (608) 264-5528; e-mail dfontani@facstaff.wisc.edu].

† University of Wisconsin—Madison.

‡ U.S. Department of Agriculture.

10.1021/jf0104331 CCC: $20.00
Published on Web 09/27/2001

globulin in vitro, and its activity was modulated by an endogenous inhibitor (*17*).

This paper reports how, using one- and two-dimensional PAGE with gels that contained incorporated gelatin as substrate, we have investigated the biochemical properties and possible roles of a group of metallopeptidases that have been extracted from barley green malt. To the best of our knowledge this is the most detailed study of cereal seed metallopeptidases that has been carried out.

MATERIALS AND METHODS

Plant Material. Barley crop was grown in 1998 in Idaho under irrigation. Green malt was obtained by steeping barley kernels (*Hordeum vulgare* cv. Morex) at 16 °C to 45% (w/w) moisture for ~36 h, by alternating 4 h of wet steep with 4 h air rests. The steeped seeds were germinated in the dark for 5 days at 17 °C and near 100% humidity (*18*). The green malt was stored at −20 °C until extracted.

Enzyme Extraction and Purification. The extraction and all purification steps were conducted at 4 °C. Barley green malt (typically 100 g) was extracted in 2 volumes (milliliters per gram of fresh weight) of 50 mM sodium acetate buffer, pH 4.7, that contained 2 mM cysteine. The sample was ground in a Waring blender for 1 min and then in a Polytron homogenizer (Brinkmann, Lucerne, Switzerland) for 2 min. The homogenate was centrifuged for 15 min at 14500*g*, and the supernatant was filtered through a double layer of cheesecloth. This crude extract was divided into aliquots and kept frozen at −18 °C until needed.

An 80-mL aliquot of the crude extract was dialyzed in 3000 molecular weight cutoff tubing overnight. The dialysis was against 1 L of 10 mM disodium phosphate that had been titrated to pH 6.0 with citric acid (citrate−sodium phosphate buffer). After it was centrifuged for 15 min at 14500*g*, the supernatant was loaded onto a 2.0 × 4.5 cm DEAE (DE52, Whatman, Maidstone, U.K.) anion-exchange chromatography column that had been equilibrated with 10 mM citrate−sodium phosphate buffer, pH 6.0. The column was washed with the same buffer until the 280-nm absorbance returned to the baseline. The bound material was then eluted with a 0.01−0.3 M linear gradient of the citrate−sodium phosphate buffer (500-mL total volume). Fractions (8.5 mL) from the column were collected, and their activities were assayed by PAGE on gelatin-containing gels. Those fractions that contained the activity of interest were pooled.

Proteins in the pooled fractions were precipitated by adding ammonium sulfate to a concentration of 40% (w/v) and incubating for 15 min on ice. The sample was centrifuged for 10 min at 14500*g*, after which time the precipitated proteins were resuspended in 2 mL of 25 mM histidine−HCl, pH 6.2, and dialyzed at 4 °C against 1.5 L of the same buffer.

The concentrated dialyzed sample was applied to a 1.2 × 38.0 cm PBE-94 chromatofocusing column (Pharmacia Biotech, Uppsala, Sweden) that had been equilibrated with 25 mM histidine−HCl, pH 6.2. The sample was eluted with Polybuffer 74−HCl, pH 4.0, over a 480-mL pH gradient that ranged from 6.2 to 4.0. The gradient was generated as recommended by the Polybuffer 74 manufacturer.

The fractions that did not elute from the column with this gradient were displaced with 0.5 M NaCl after the pH gradient was completed. The chromatofocusing eluent was collected in 4.2-mL fractions. These fractions were analyzed and the active samples pooled. In this paper, these will be referred to as MP (for "metallopeptidase"), because they were inhibited by metal-chelating agents.

Electrophoretic Enzyme Assays. Electrophoretic separations were performed according to the method of Wrobel and Jones (*9*). Native PAGE was carried out in 11% polyacrylamide gels containing 0.1% gelatin (11% PAGE-gelatin gels). Denaturing 6.6% polyacrylamide gels, which contained 0.1% gelatin (6.6% PAGE-gelatin gels), were prepared using a modification of the method described by Wrobel and Jones (*19*) that included 4% SDS in the gel solutions and electrode buffer. After the electrophoretic separation, the gels were briefly rinsed with distilled water and placed in the incubation buffer, which was 0.1 M Tris-HCl, pH 8.0, unless specified otherwise. The gels were not incubated with Triton X-100.

When two-dimensional PAGE was utilized, native or denaturing PAGE analyses were run with gelatin-containing gels as the second dimension separation, using a modification of the method by Zhang and Jones (*10*). The first dimension separation was IEF in tube gels (1.5 mm × 7.5 cm) that contained 5% acrylamide−0.3% bis(acrylamide) and ampholines in the pH range 2.5−7.0 or 3.5−10.0. The cathodic buffer was 0.1 M NaOH, and the anodic buffer was 0.1 M H_3PO_4 (or 0.1 M acetic acid for the 3.5−10.0 pH gradient).

After the electrophoretic separation, the PAGE-gelatin gels (in the presence or absence of SDS) were incubated overnight at 40 °C in 100 mL of 0.1 M Tris-HCl buffer, pH 8.0, unless indicated otherwise. The gel development was stopped by replacing the buffer with 0.1% amido black in water/methanol/acetic acid (6:3:1). After the gels were destained in water/methanol/acetic acid (6:3:1), the proteolytic activities were detected as clear spots, where the substrate had been degraded, against the blue-stained protein background.

Determination of the Optimum pH. The optimum pH of the peptidases was determined using 6.6% PAGE-gelatin gels. Each of the lanes of a minigel (6 × 8 cm) was loaded with 20 μL of MP. After electrophoresis, the gels were cut into strips and the individual strips were incubated overnight in the following buffers: 0.1 M citrate−sodium phosphate at pH 3.0, 4.0, 5.0, 6.0, and 7.0; 0.1 M Tris-HCl at pH 7.0 and 8.0; 0.1 M ammonium carbonate at pH 8.0, 9.0, and 10.0. The activities on each of the strips were assessed by staining with amido black, as described previously. The activity bands on the gels were quantified visually and with the Kodak Digital Science 1D image analysis software (Eastman Kodak Co., Rochester, NY).

Studies with Reducing Agents and Inhibitors. The effects of reducing agents on the gelatin-hydrolyzing activities of the MP enzymes were studied after the peptidases were separated on 6.6% PAGE-gelatin gels in the presence of SDS. The chemicals tested were β-mercaptoethanol (β-ME) (10 and 20 mM), cysteine (10 and 20 mM), and dithiothreitol (DTT) (5 and 10 mM). The gels were developed in 0.1 M Tris-HCl buffer, pH 8.0, that contained the reducing agent to be tested.

Inhibition studies with class-specific synthetic inhibitors were also carried out using the electrophoretic enzyme assay. The MP peptidases were separated on 6.6% PAGE-gelatin gels, in the presence of SDS. After electrophoresis, the individual gel strips were incubated overnight in 12 mL of 0.1 M Tris-HCl buffer, pH 8.0, containing the following concentrations of synthetic inhibitors: 10 or 20 mM EDTA (from a 0.2 M stock solution, dissolved in water), 10 or 20 μM E-64 (1 mM stock solution, in water), 1 or 5 mM o-phen [0.4 M stock solution, in dimethyl sulfoxide (DMSO)], 20 or 40 μM pepstatin A (1 mM stock solution, in ethanol), and 10 or 20 mM phenylmethanesulfonyl fluoride (PMSF) (0.4 M stock solution, in DMSO).

Recovery of Activity by the Addition of Metal Ions. The recovery of the enzymatic activities in the presence of metal ions, after inhibition by EDTA, was also investigated. The MP peptidases were separated on 6.6% PAGE-gelatin gels, in the presence of SDS. The gel strips were then incubated for 20 min in the presence of 0.1 mM EDTA, rinsed briefly three times with ultrapure water, and developed in buffer that contained either $ZnCl_2$, $Co(CH_3CO_2)_2$, or $Mn(CH_3CO_2)_2$. The metal ion concentrations tested were 0.1, 0.5, 1, 5, and 7.5 mM.

Localization and Time of Appearance of the MP Enzymes during Barley Germination. The locations of the MP peptidases in germinated barley seeds and when they appeared during germination were studied using 11% PAGE-gelatin gels.

Fifty 4-day-germinated dehusked barley seeds were dissected by hand, using sterile scalpel blades, into root, axis, scutellum, starchy endosperm, and aleurone fractions. The isolated tissues were frozen in liquid nitrogen and ground with a mortar in microfuge tubes. They were extracted with 2

volumes (milliliters per gram of fresh weight) of 50 mM sodium acetate buffer, pH 4.7, containing 2 mM cysteine, using a Ultra-Turrax T8 homogenizer (IKA Labortechnik, Staufen, Germany). Aliquots (15 μL each) of the tissue extracts were analyzed on one- and two-dimensional 11% PAGE-gelatin gels.

Fifty grams each of ungerminated, out-of-steep, and 1-day-germinated barley was extracted with 2 volumes (milliliters per gram of fresh weight) of 50 mM sodium acetate buffer, pH 4.7, containing 2 mM cysteine, in a Waring blender with four 1-min spins alternating with 1-min rests. The activities of 15-μL aliquots of the extracts were then analyzed on two-dimensional 11% PAGE-gelatin gels.

SDS-PAGE. The electrophoresis of denatured proteins was conducted in 12% polyacrylamide gels that contained SDS, according to the method of Laemmli (*20*). The samples were denatured by heating for 3 min at 100 °C in sample buffer that contained 4% (w/v) SDS and 10% (v/v) β-ME. After electrophoresis, the gels were fixed for 30 min in 12% (w/v) TCA and stained with silver nitrate according to the method of Oakley et al. (*21*), as modified by Eschenbruch and Burk (*22*).

Isolation of D Hordein. Hordeins were extracted from 40 mg of barley flour at 60 °C with 1 mL of 55% (v/v) 2-propanol/2% (v/v) β-ME according to the method of Heisel et al. (*23*). The extract was centrifuged for 3−5 min at 14200*g*, and the supernatant comprised the 'total hordein' preparation.

The D hordein fraction was isolated by subjecting this total hordein preparation to RP-HPLC on a Supelco (Supelco Park, Bellefonte, PA) Discovery C18 column (0.46 × 5.0 cm) that was eluted at a flow rate of 1 mL/min at room temperature. The elution solvents were (A) 0.1% TFA in water and (B) 0.1% TFA in acetonitrile. The hordein preparation was applied to a column that was equilibrated with 10% solvent B and, after 3 min, the concentration of solvent B was increased linearly to 100% over 20 min. The elution was monitored at 280 nm with a Shimadzu SPD-10A VP (Shimadzu Corp., Kyoto, Japan) diode array detector. The D hordein fraction eluted at 42% acetonitrile and was collected and freeze-dried twice before use.

In Vitro Hydrolysis of Hordeins. The D hordein storage protein was partially purified by RP-HPLC as described and was tested as a substrate for the MP enzymes. A 500-μL aliquot of the D hordein sample was freeze-dried twice and resuspended in 10 μL of 55% 2-propanol. This sample was mixed with 200 μL of MP at 40 °C in a water bath. After 0, 2, 4, 8, and 24 h, 35 μL of the reaction mixture was removed and 15 μL of this hydrolyzed sample was added to 15 μL of SDS sample buffer and analyzed on 12% SDS-PAGE gels. The remaining 20 μL of sample was subjected to analysis by RP-HPLC, as described. Control reactions were also run, in which the MP sample was replaced by the buffer that was used to elute it from the chromatofocusing column (Polybuffer 74−HCl, pH 4.0, with added 0.5 M NaCl).

A similar set of reactions was run in which the D hordein was mixed with 75 μL of MP and 45 μL of 0.1 M Tris-HCl buffer, pH 8.0. After 0, 40, 80, and 120 min, 35 μL of the reaction mixture was removed, added to SDS sample buffer, and analyzed on 12% SDS-PAGE gels. In the control reaction the MP sample was replaced by Polybuffer 74−HCl, pH 4.0. To test for inhibition, the MP enzymes were preincubated with inhibitors (or with DMSO, the solvent for some of the inhibitors) for 20 min at 40 °C, and the reaction was started by adding the D hordein substrate. The inhibitors that were tested and their concentrations were as follows: EDTA, 20 mM; E-64, 0.1 mM; o-phen, 10 mM; pepstatin A, 20 μM; and PMSF, 10 mM.

RP-HPLC Analysis of the D Hordein Hydrolysis by MP. Analyses of the D hordein hydrolysis samples were performed by RP-HPLC on a Supelco Discovery C18 column (0.46 × 5.0 cm), using the same method that was used to purify the protein.

RESULTS

Purification of the MP Enzymes. When a dialyzed extract of germinated barley was applied to and eluted from a DEAE column, the MP activities eluted between the concentrations of 40 and 70 mM citrate−sodium phosphate buffer (Figure 1A).

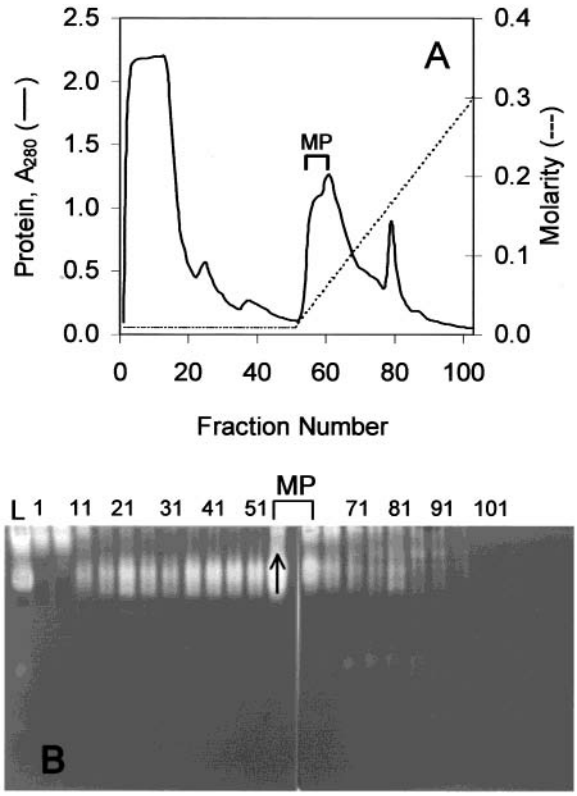

Figure 1. Ion-exchange fractionation of the MP peptidases (further characterization has proven that these were metallopeptidases; see Figure 4): (A) anion-exchange chromatography of a germinated barley extract on a DEAE column that was eluted with citrate−sodium phosphate buffer, at pH 6.0; (B) assay of the fractions eluted from the ion-exchange column. Every fifth fraction was analyzed with gelatin-containing PAGE gels that were developed overnight (16 h) at pH 6.0. The fractions that were pooled for further purification are indicated by the brackets and the MP enzymes by the arrow. Lane L was loaded with 20 μL of the unseparated crude sample.

The DEAE fractions were analyzed for their abilities to hydrolyze gelatin substrate that was incorporated into native 11% polyacrylamide gels, and the results are shown in Figure 1B.

The MP activities appeared as a slowly migrating band that eluted from the DEAE column together with major contaminating gelatinolytic activities that migrated further into the gel. The MP enzymes were discriminated from other low-mobility fractions that did not bind to the column and had basic isoelectric points (p*I* values) by two-dimensional gelatin PAGE analyses (results not shown). Several contaminant proteases were separated from the MP group by the DEAE column. Some (lanes 1−51) did not bind to the column, and others (lanes 66−101) eluted at different points along the gradient.

The minimum ammonium sulfate concentration that precipitated the metallopeptidase activities was 40%, so this concentration was used to precipitate the activities of interest that were collected from the DEAE column.

Further purification of the MP activities was achieved by separation on a PBE-94 chromatofocusing column (fractions 56−61 from DEAE). Preliminary data from

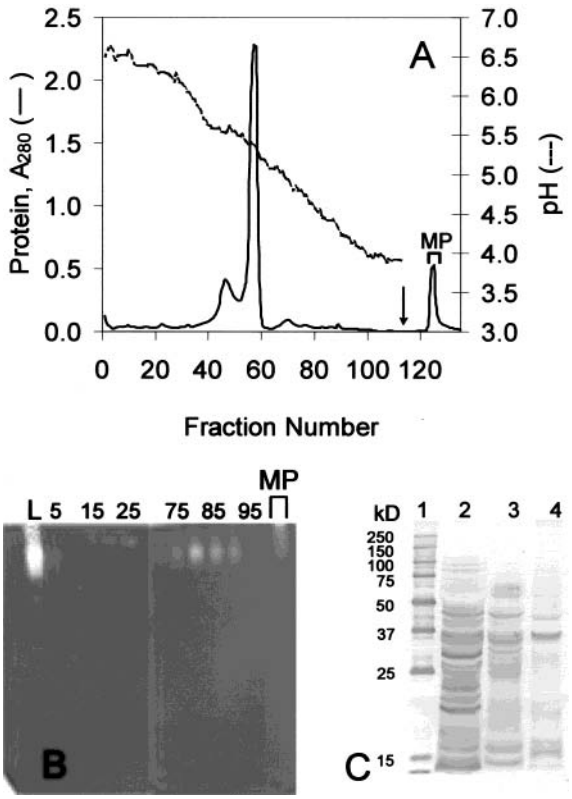

Figure 2. Chromatofocusing separation of the MP peptidases (the enzymes were assigned to the class of metallopeptidases with later studies; see Figure 4): (A) chromatofocusing of the pooled fractions from Figure 1A on a PBE-94 column. The arrow indicates where elution with 0.5 M NaCl began. The brackets indicate the fractions comprising the MP activities. (B) Electrophoretic enzyme assay of the fractions eluted from the PBE-94 column. Every fifth fraction was analyzed by overnight incubation at pH 6.0 in gelatin-containing PAGE; lane L contained a sample of the preparation as loaded on the column. The lane that contained the partially purified MP fractions is indicated by the brackets (MP). (C) 12% SDS−PAGE of the MP peptidases containing fractions during the purification steps: (lane 1) precision molecular weight standards (Bio-Rad); (lane 2) malted barley extract; (lane 3) pooled fractions from the DE-52 column; (lane 4) pooled fractions from the PBE-94 chromatofocusing column.

two-dimensional PAGE-gelatin gels had indicated that the MP enzymes had p*I* values that ranged between 4.5 and 5.5. To separate these, a pH gradient that ran from pH 6.2 to pH 4 was generated on the PBE-94 column (Figure 2A).

The proteins that bound to the column at pH 6.2 were eluted as the decreasing pH gradient reached their isoelectric points except for the MP peptidases. They bound to the column strongly and were not released until 0.5 M NaCl was added to the Polybuffer 74−HCl at the end of the gradient, after the pH had reached 4.0. The metallopeptidases apparently had p*I* values of 4.5−5.5 but did not elute from the chromatofocusing column at that pH, indicating that they bound to the column nonspecifically. This binding provided a convenient way to separate the peptidases from the contaminants.

As shown in Figure 2B, chromatofocusing readily separated the MP enzymes from the contaminating gelatinolytic activities that had similar p*I* values (observations from two-dimensional gelatin PAGE analyses, not shown) and from a second contaminant that had a higher p*I* value but that did not bind to the PBE-94 column (lanes 20−25). The proteins that eluted in fractions 50−60 did not contain any gelatin-hydrolyzing activities. Figure 2C shows how the MP peptidases were purified during the various purification steps, as determined by SDS-PAGE. Due to the complexity of the sample, which comprised a mixture of metallopeptidases, specific molecular weights could not be assigned to each of the MP enzymes.

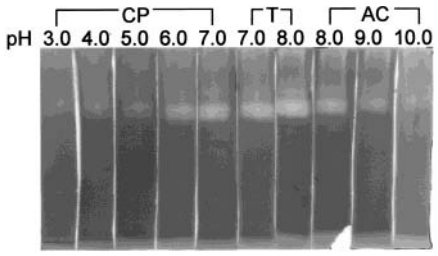

Figure 3. Effect of pH on the MP activities. The MP enzymes were separated on 6.6% polyacrylamide gels containing gelatin substrate. The activity of each gel strip was developed after overnight incubation at the pH and in the buffers that are indicated above the gels. CP, citrate−sodium phosphate buffer; T, Tris-HCl buffer; AC, ammonium−carbonate buffer.

Characterization of the MP Enzymes. Because the purified MP preparation contained multiple metallopeptidase activity forms, the characterization tests were done using the electrophoretic enzyme assay rather than an "in solution" assay. The gel assay method allowed the separation and identification of individual enzymes, making it possible to verify whether all of the enzymes shared the same biochemical properties. An "in solution" assay would not have discriminated among the activities of the multiple components, making it impossible to determine whether a particular result was due to the activities of all, or just a portion, of the MP enzymes.

The pH optimum of MP was evaluated by testing its activity between pH 3.0 and 10.0, using three different buffer systems that overlapped at pH 7.0 and 8.0 (Figure 3). The enzymes were weakly active at pH 3.0−5.0 and were most active between pH 7.0 and 8.0. The optimal gelatin-hydrolyzing activity was detected at pH 8.0, in the presence of Tris-HCl. The pH 7.0 and 8.0 activities were slightly affected by the presence of the citrate−sodium phosphate and ammonium carbonate buffers, because the hydrolyzed areas on the gel were somewhat diminished in these buffers relative to those seen when the gel was incubated in Tris-HCl.

The peptidase classes to which the MP enzymes belonged were determined by measuring their activities in the presence of class-specific inhibitors (Figure 4). The only compounds that inhibited the enzyme activities were EDTA and o-phen, two metal-chelating agents. Neither E-64 (an inhibitor of cysteine peptidases), pepstatin A (an inhibitor of aspartic peptidases), nor PMSF (an inhibitor of serine peptidases) affected the activities, indicating that all of the MP peptidases were metallopeptidases. To confirm that all of the MP enzymes were metal-dependent enzymes, they were separated with a two-dimensional system in which the PAGE gel contained gelatin, providing increased resolution of the enzyme mixture. With this method, the multiple MP components were well separated into three major and six or so minor activity spots (Figure 5A) that had isoelectric points in the range of 4.5−5.5.

The activities on the gels were also developed in pH 8.0 buffer that contained either o-phen or PMSF (Figure

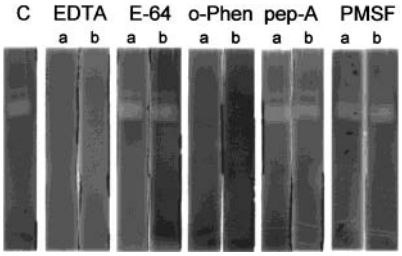

Figure 4. Effect of class-specific inhibitors on the MP activities. Lane C was developed overnight in buffer that containined no inhibitors. The other gel strips were developed overnight in the presence of the inhibitors indicated. The inhibitor concentrations were as follows: EDTA, 10 and 20 mM (lanes a and b, respectively); E-64, 10 and 20 μM; o-phen, 1 and 5 mM; pepstatin A, 20 and 40 mM; PMSF, 10 and 20 mM. Twenty microliters of MP was applied to each strip.

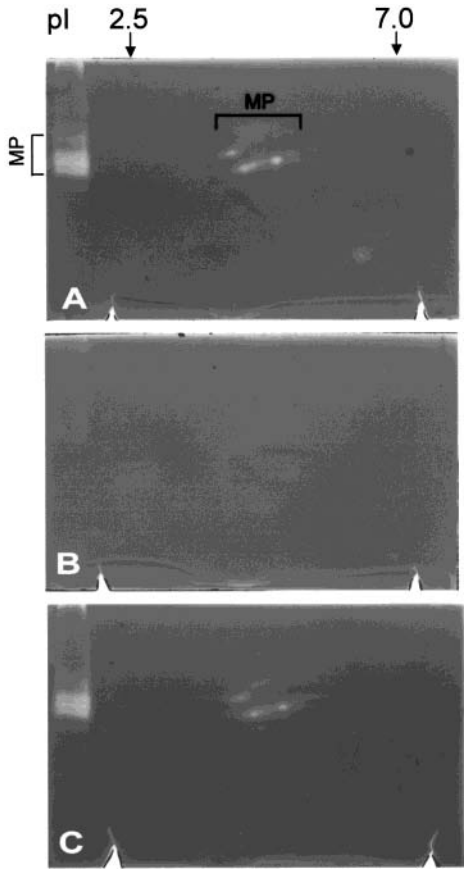

Figure 5. Two-dimensional separations of the MP peptidases on gelatin-containing gels: (A) gel was developed at pH 8.0 in the absence of inhibitor; (B) gel developed in the presence of 1 mM o-phen; (C) gel developed in 10 mM PMSF. Twenty microliters of MP was applied to each gel. All gels were incubated overnight. The left-hand lane was loaded with sample after the IEF had finished and thus was separated only by PAGE.

5B,C). These gels confirmed that all of the activity spots that corresponded to the MP enzymes were equally inhibited by o-phen and that they were all unaffected by PMSF. The activities on the control gel (Figure 5A) were developed in the absence of inhibitors.

The effects of reducing agents on the MP activities were tested by adding β-ME, DTT, or cysteine to the incubation buffers used for developing the enzymes that had been separated on 6.6% PAGE-gelatin gels. The activities of the MP peptidases were all inhibited by each of these reducing agents (Figure 6).

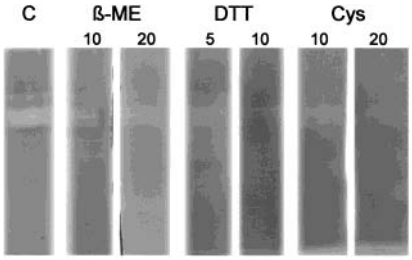

Figure 6. Effects of reducing agents on the gelatin-hydrolyzing activity of the MP enzymes. The activities on strip C (control) were developed in 0.1 M Tris-HCl buffer, pH 8.0. The other strips were developed in the presence of the reducing agents indicated: β-ME, 10 and 20 mM; DTT, 5 and 10 mM; cys, 10 and 20 mM. All gels were incubated overnight.

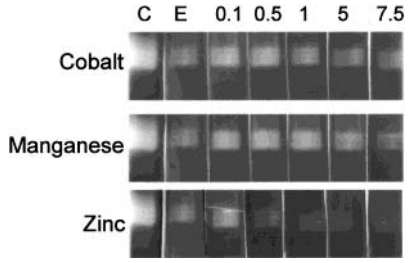

Figure 7. Restoration of the proteolytic activities of EDTA-inactivated MP enzymes by metal ions. Strips C and E were control gels that were developed in 0.1 M Tris-HCl buffer, pH 8.0, in the absence and presence of 0.1 mM EDTA, respectively. The remaining strips were incubated in EDTA, rinsed, and developed in the presence of the indicated concentrations of metal ions. All gels were incubated overnight.

The minimum concentration of EDTA that gave any inhibition of the MP activities was 0.1 mM (data not shown). Tests were then run to determine which, if any, metal ions could reactivate EDTA-inhibited enzymes. The MP enzymes were separated on PAGE-gelatin gels, which were incubated for 30 min in buffer that contained 1 mM EDTA. The EDTA was then rinsed from the gels, and the enzyme activities were developed in the presence of various concentrations of cobalt, manganese, or zinc (Figure 7). All of the metal ions tested reactivated, to various extents, the enzymatic activities of the peptidases, with the maximum recoveries occurring at concentrations of 0.1 mM (cobalt and zinc) or 0.5 mM (manganese). When higher metal ion concentrations were tested, they did not further stimulate the activities but, instead, had an inhibitory effect. This inhibition was especially strong with Zn^{2+}.

Localization of MP in Barley Seed Tissues and Its Appearance during Germination. To help clarify the role played by MP endopeptidases in germinated barley, the locations in which they reside in the seed tissues and the times when they appear during germination were studied.

When the seed tissues from 4-day-germinated barley were separated and extracted and their activities analyzed using one-dimensional 11% PAGE-gelatin gels, proteolytic activities with migration rates similar to those of the MP enzymes were present in the starchy endosperm, aleurone, scutellum, and rootlet tissues (Figure 8A). Because one-dimensional gels cannot separate activities with similar migration rates but different p*I* values, the samples were also analyzed on two-dimensional gels. This system showed that the MP activities were mainly localized in the aleurone tissue (Figure 8B) with little presence in the scutellum. None of the other tissues contained proteinase activities that

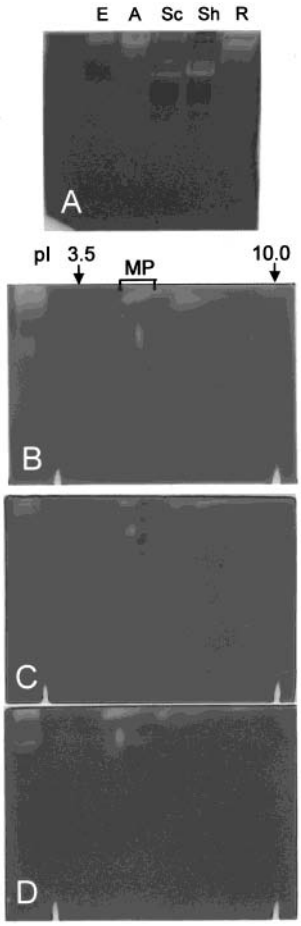

Figure 8. Localization of the MP peptidase in seed tissues and their formation during barley germination: (A) separation of crude extract from 4-day-malted seed tissues on one-dimensional 11% PAGE gelatin gels; (B) separation of crude extract from 4-day-malted seed aleurone on two-dimensional 11% PAGE gelatin gels; (C, D) separation of crude extract from resting and 1-day-germinated barley seeds, respectively, on two-dimensional 11% PAGE gelatin gels. The tissue extracts applied on the gel in panel A were as follows: E, starchy endosperm; A, aleurone; Sc, scutellum; Sh, shoot; R, root. All gels were incubated overnight.

migrated to the MP area of the gels. Only very low MP activity levels were detected in resting (Figure 8C) or out-of-steep seeds, but the activities increased sharply during the first day of germination (Figure 8D). Their activities were still strong through the fourth day of germination (data not shown).

In Vitro Hydrolysis of Hordeins by MP. To ascertain the contribution of the MP enzymes to the protein degradation that occurs during germination and malting, it was necessary to determine whether they played a role in hydrolyzing any of the barley storage proteins in green malt. In a preliminary study, when an extract of barley storage proteins (hordeins) was used as a substrate for the MP peptidases, the D component of this hordein preparation, but not the other components, was readily degraded (data not shown). It is notable that this reaction was carried out at pH 4.0, far from the optimal pH of the MP peptidases as determined on the substrate gelatin. The same band persisted throughout the control (no MP added) reaction, indicating that it was the MP enzymes that hydrolyzed the D hordein.

To more closely monitor the hydrolysis of the D hordein by the MP enzymes, this protein was isolated,

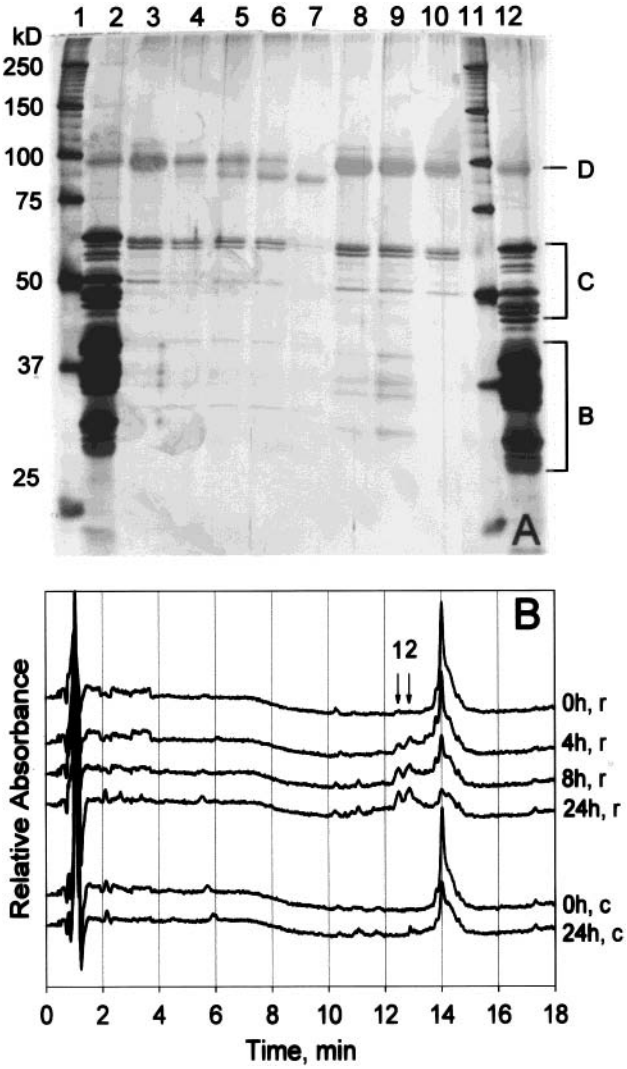

Figure 9. In vitro hydrolysis of a D hordein preparation by the MP enzymes: (A) SDS-PAGE separation of the hydrolysis products. The D hordein was incubated in the presence of MP (control, lanes 3−7) or in buffer only (lanes 8−10). Aliquots were removed from the reaction at appropriate times and analyzed. Lanes 1 and 11 are Sigma Precision molecular weight standard; lanes 2 and 12 are total hordein preparation; lanes 3−7 are reaction aliquots hydrolyzed for 0, 2, 4, 8, or 24 h, respectively; lanes 8−10 are control aliquots (no MP) at times 0, 8, and 24 h, respectively. The hordein classes are indicated on the right side of the figure. (B) RP-HPLC analysis of the products formed by hydrolyzing the D hordein preparation with the MP enzymes. The peptides that formed are indicated by the arrows. The "r" next to a reaction time indicates an aliquot removed from the reaction mixture; a "c" indicates the analyzed sample was from the control reaction (no MP enzymes added).

partially purified, and used as substrate for the peptidases. When the hydrolysis products at various times were separated on 12% SDS-PAGE, a peptide that migrated more rapidly than the D hordein formed after 2 h of incubation, and the D hordein band had completely disappeared after 24 h of reaction (Figure 9A). RP-HPLC analysis of the D protein degradation products (Figure 9B) indicated that two peptides had formed, whose elution times from the C-18 column were earlier than that of the original protein. The 24-h control reactions of Figure 9A,B both showed that some of the D hordein disappeared from the reaction mixture, even in the absence of added enzyme. Additional observations (not reported here) had indicated that some of the

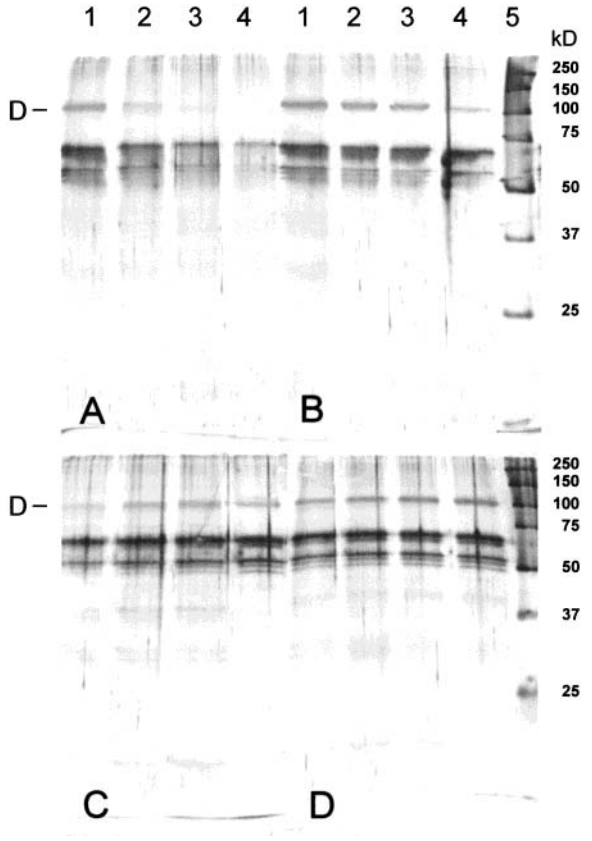

Figure 10. In vitro hydrolysis of D hordein by the MP peptidases at pH 8.0 and its inhibition by EDTA and o-phen. The D hordein was incubated in the presence of MP (A), in buffer only (B), with MP in the presence of EDTA (C), and with MP in the presence of o-phen (D). Reaction aliquots were removed at appropriate times and analyzed. Lanes 1−4 are reaction aliquots removed after 0, 40, 80, and 120 min, respectively; lane 5 shows precision molecular weight standards (Bio-Rad). The D hordein band is indicated.

hordeins had begun to precipitate from the reaction after only 2 h of incubation. Although no detectable precipitate was visible, both the SDS-PAGE and HPLC analyses of the control reaction products showed that some of the storage proteins disappeared progressively from the control reactions during incubation but that there was no concomitant appearance of degradation products. However, in experiments that were carried out in the presence of MP, the formation of hydrolysis products was clearly observed and the rate of disappearance of the D hordein by hydrolysis was obviously greater than that due to the spontaneous precipitation of the proteins.

When the reaction was carried out in buffer at pH 8.0, the pH optimum of the peptidase preparation, the D hordein was hydrolyzed more rapidly (Figure 10A), the protein being totally digested after only 40 min of incubation. Tests in which protease class-specific inhibitors were added to the reaction mixtures showed that the hydrolysis still occurred when E-64, pepstatin A, and PMSF were present (not shown) but not in the presence of EDTA or o-phen (Figure 10C,D), both of which specifically inhibit metallopeptidases.

DISCUSSION

The metallopeptidases comprise a complex group of enzymes. They are widely distributed among prokaryotes and eukaryotes, and they share many structural similarities (*24*). Despite the many studies that have been devoted to investigating these enzymes in animals and bacteria, very little is known about the plant metallopeptidases, especially those present in cereal seeds. Plant seed metallopeptidases have been purified from buckwheat (*16*), and it has been reported that they occur in pumpkin (*25*), maize (*26*), wheat (*27*), and barley (*10*, *14*, *15*). In barley, metal-activated endopeptidases were responsible for 31% of the total gelatin hydrolytic activity of barley and for 9% of the activity of a green malt preparation (*14*). Five high molecular weight gelatin-hydrolyzing metallopeptidases were demonstrated in 4-day-germinated green malt by Wrobel and Jones (*15*). Some of the biochemical properties of those enzymes (the effects of EDTA, metal ions, and DTT) resembled those of the MP peptidases, but they were only poorly separated and characterized, so just these few comparisons can be made. No detailed study of cereal seed metallopeptidases had been reported, however, and their roles in resting and/or germinating seeds were still unknown.

In this study, the MP enzymes were purified to the point that no other gelatin-hydrolyzing activity was present in the preparation. They were considered to be functional isoenzymes because of their common abilities to digest gelatin and because their activities were affected equally by inhibitors, reducing agents, and metal ions. In addition, they migrated very similarly on gelatin-incorporated PAGE gels in the presence of SDS and could be resolved well only by using a two-dimensional electrophoretic system. With this method, they separated into three major and approximately six minor activity spots, all of which had acidic p*I* values that fell in the range of 4.5−5.5. Unlike other enzymes that have been studied with this method (*28*), the activities of these peptidases, after PAGE in the presence of SDS, were recovered without the necessity of having to rinse the gels in Triton X-100. This indicates either that the enzymes did not lose their activities on binding SDS or that incubating the gel in the development buffer was sufficient for removing the SDS and recovering the activities. The MP enzymes were totally inhibited by the metal-chelating agents EDTA and o-phen, as were the majority of the metallopeptidases that have been purified from bacteria and animals.

Once inhibited by EDTA, the activities of the MP enzymes were partially restored by the addition of the metal ions Co^{2+}, Mn^{2+}, and Zn^{2+}. Manganese was the most effective ion for restoring the activities, at most concentrations. Zinc ions partially restored the MP activities, but only at concentrations <0.1 mM; an excess of zinc inhibited all of the activities. No specific study was conducted to characterize which metal ion was involved in the catalytic mechanism of the isozymes. Nevertheless, the facts that the MP group was inhibited by low concentrations of zinc and activated by even lower concentrations and that most of the metallopeptidases of bacterial and animal origin contain zinc in their active centers (*29*) lead us to conclude that the MP activities are also probably zinc-dependent metallopeptidases.

The properties of these barley MP enzymes (p*I* values, pH optima, and inhibition characteristics) are similar to those of other metallopeptidases that have been studied. Belozersky et al. (*16*) purified a metallopeptidase from resting buckwheat seeds that had an M_r of 34000 kDa and a pH optimum against the native

substrate (buckwheat seed globulin) of 8.0−8.2 and that required zinc for its activity. That enzyme carried out a limited in vitro proteolysis of the buckwheat storage globulin and was believed to initiate the hydrolysis of the 13 S globulin in vivo. Metallopeptidases that were able to digest storage proteins have also been found in the resting seeds of pumpkins (*25*) and soybeans (*30*) but, prior to this study, no metal-requiring enzymes from a cereal seed had been shown to hydrolyze storage proteins. This MP enzyme group readily hydrolyzed the D hordein barley storage protein in vitro. Previous studies on the development times of the gelatinolytic activities of barley (*10*, *31*) identified the MP enzymes on two-dimensional PAGE-gelatin gels as three activity spots, namely, A5, A6, and A7. The separation method employed for that study, which used 12% polyacrylamide gels developed at pH 4.8 in the presence of cysteine, did not separate all of the multiple components in the MP group. The activities labeled A5, A6, and A7 were described as being present in all tissues of 4-day-germinated barley seeds, with the highest concentration found in the aleurone. In our experiments with resting barley samples and 1-day- through 4-day-germinated seeds, with separation gels that were developed at pH 8.0, the enzymes increased greatly during the first day of germination, and their activities were still maximal on the fourth day of malting. There is strong evidence that peptidases that are inhibited by the metal-chelating agent o-phen affected the solubilization of barley storage proteins during the mashing stage of the brewing process (*32*). These observations suggest that metallopeptidases are probably involved in the in vivo hydrolysis of barley storage proteins during malting and mashing.

The MP peptidases differ from the buckwheat metallopeptidase purified by Belozersky et al. (*16*) because the barley enzymes appear (either by de novo synthesis or by activation) during germination and are scarcely present in resting seeds. This suggests that they might participate in the bulk hydrolysis of storage proteins rather than in initiating the protein degradation. The fact that the barley peptidase activities reached their maximum levels on the fourth day of germination also agrees with this possibility. However, we did not find any evidence that the MP enzymes were present in the starchy endosperm tissue, where the majority of the barley storage proteins are localized. In fact, the MP peptidases were mainly found in the aleurone layer of the germinated barley seeds, which would preclude their playing a role in the bulk hydrolysis of storage proteins.

Sunblom and Mikola showed in 1972 (*33*) that isolated aleurone layers released a metal-activated enzyme that hydrolyzed gelatin at pH 7.0 when they were treated with gibberellic acid. These results are consistent with the hypothesis that some metallopeptidases are synthesized de novo at the beginning of germination under the stimulation of gibberellic acid that is released by the embryo. The metallopeptidases they studied digested gelatin over a wide range of pH values, as do the MP enzymes that we have isolated. However, if the MPs were the same enzymes as those isolated by Sundblom and Mikola, they should have been present in the endosperm, to which they would have moved when they were secreted from the aleurone. Further studies are needed to provide conclusive evidence that will better define the roles of these metallopeptidases in hydrolyzing the storage proteins.

ABBREVIATIONS USED

β-ME, β-mercaptoethanol; DTT, dithiothreitol; E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)-butane; IEF, isoelectring focusing; o-phen, 1,10-phenanthroline; PMSF, phenylmethanesulfonyl fluoride; MP, metallopeptidases.

ACKNOWLEDGMENT

We thank Laurie A. Marinac for her excellent technical assistance and Eddie Goplin for malting the barley.

LITERATURE CITED

(1) Jacobsen, J. V.; Varner, J. E. Gibberellic-acid induced synthesis of protease by isolated aleurone layers of barley. *Plant Physiol.* **1967**, *42*, 1596−1600.
(2) Shewry, P. R. Barley seed proteins. In *Barley. Chemistry and Technology*; MacGregor, A. W., Bhatty, R. S., Eds.; American Association of Cereal Chemists: St. Paul, MN, 1993; pp 131−197.
(3) Poulle, M.; Jones, B. L. A proteinase from germinating barley. I. Purification and some physical properties of a 30 kD cysteine endoproteinase from green malt. *Plant Physiol.* **1988**, *88*, 1454−1460.
(4) Phillips, H.; Wallace, W. A cysteine endopeptidase from barley malt which degrades hordein. *Phytochemistry* **1989**, *28*, 3285−3290.
(5) Koehler, S. M.; Ho, T.-H. D. A major gibberellic acid induced barley aleurone cysteine proteinase which digests hordein. *Plant Physiol.* **1990**, *94*, 251−258.
(6) Zhang, N.; Jones, B. L. Purification and partial characterization of a 31-kDa cysteine endopeptidase from germinated barley. *Planta* **1996**, *199*, 565−572.
(7) Sarkkinen, P.; Kalkkinen, N.; Tilgmann, C.; Siuro, J.; Kervinen, J.; Mikola, L. Aspartic proteinase from barley grain is related to mammalian lysosomal cathepsin D. *Planta* **1992**, *186*, 317−323.
(8) Zhang, N.; Jones, B. L. Polymorphism of aspartic proteinases in resting and germinating barley seeds. *Cereal Chem.* **1999**, *76*, 134−138.
(9) Wrobel, R.; Jones, B. L. Appearance of endoproteolytic enzymes during the germination of barley. *Plant Physiol.* **1992**, *100*, 1508−1516.
(10) Zhang, N.; Jones, B. L. Characterization of germinated barley electrophoretic enzymes by two-dimensional gel electrophoresis. *J. Cereal Sci.* **1995**, *21*, 145−153.
(11) Terp, N.; Thomsen, K. K.; Svendsen, I.; Davy, A.; Simpson, D. J. Purification and characterization of hordolisin, a subtilisin-like serine endopeptidase from barley. *J. Plant. Physiol.* **2000**, *156*, 468−476.
(12) Mitsuhashi, W.; Koshiba, T.; Minamikawa, T. Separation and characterization of two endopeptidases from cotyledons of germinating *Vigna mungo* seeds. *Plant Physiol.* **1986**, *80*, 628−634.
(13) Qi, X.; Wilson, K. A.; Tan-Wilson, A. Characterization of the major protease involved in the soybean β-conglycinin storage protein mobilization. *Plant Physiol.* **1992**, *99*, 725−733.
(14) Enari, T.-M.; Mikola, J. Characterization of the soluble proteolytic enzymes of green malt. In *European Brewery Convention Proceedings of the 11th Congress, Madrid, Spain*; Elsevier: Amsterdam, The Netherlands, 1967; pp 9−16.
(15) Wrobel, R.; Jones, B. L. Identification and partial characterization of high M_r neutral proteinases from 4-day germinated barley seed. *J. Cereal Sci.* **1993**, *18*, 225−237.
(16) Belozersky, M. A.; Dunaevsky, Y. E.; Voskoboynikova, N. E. Isolation and properties of a metallopeptidase from buckwheat (*Fagopyrum esculentum*) seeds. *Biochem. J.* **1990**, *272*, 677−682.

(17) Dunaevskii, Y. E.; Pavlikova, E. B.; Belyakova, G. A.; Belozerskii, M. A. Physiological role of protease inhibitors in plants: two groups of active inhibitors in buckwheat seed. *Mol. Biol.* **1995**, *29*, 747−750.
(18) Jones, B. L.; Marinac, L. A.; Fontanini, D. Quantitative study of the formation of endoproteolytic activities during malting and their stabilities to kilning. *J. Agric. Food Chem.* **2000**, *48*, 3898−3905.
(19) Wrobel, R.; Jones, B. L. Electrophoretic study of substrate and pH dependence of endoproteolytic enzymes in green malt. *J. Inst. Brew.* **1992**, *98*, 471−478.
(20) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680−685.
(21) Oakley, B. R.; Kirsch, D. R.; Morris, N. R. A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. *Anal. Biochem.* **1980**, *105*, 361−363.
(22) Eschenbruch, M.; Burk, R. R. Experimentally improved reliability of ultrasensitive silver staining of protein in polyacrylamide gels. *Anal. Biochem.* **1982**, *125*, 96−99.
(23) Heisel, S. E.; Peterson, D. M.; Jones, B. L. Identification of United States barley cultivars by sodium dodecyl sulfate polyacrylamide gel electrophoresis of hordeins. *Cereal Chem.* **1986**, *63*, 500−505.
(24) Rawlings, N. D.; Barret, A. J. Evolutionary families of metallopeptidases. In *Methods in Enzymology*; Barrett, A. J., Ed.; Academic Press: San Diego, CA, 1995; Vol. 248, pp 183−228.
(25) Hara, I.; Matsubare, H. Pumpkin (*Cucurbita* sp.) seed globulin V. Proteolytic activities involved in globulin degradation in ungerminated seeds. *Plant Cell Physiol.* **1980**, *21*, 219−232.
(26) Mitsuhashi, W.; Oaks, A. Development of endopeptidase activities in maize (*Zea mays*) endosperms. *Plant Physiol.* **1994**, *104*, 401−407.
(27) Dominguez, F.; Cejudo, F. J. Characterization of the endopeptidases appearing during wheat grain development. *Plant Physiol.* **1996**, *112*, 1211−1217.
(28) Heussen, C.; Dowdle, E. B. Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing SDS and copolymerized substrate. *Anal. Biochem.* **1980**, *102*, 196−202.
(29) Auld, D. S. Removal and replacement of metal ions in metallopeptidases. In *Methods in Enzymology*; Barrett, A. J., Ed.; Academic Press: San Diego, CA, 1995; Vol. 248, pp 228−242.
(30) Bond, H. M.; Bowles, D. J. Characterization of soybean endopeptidase activity using exogenous and endogenous substrates. *Plant Physiol.* **1983**, *72*, 345−350.
(31) Zhang, N.; Jones, B. L. Development of proteolytic activities during barley malting and their localization in the green malt kernel. *J. Cereal Sci.* **1995**, *22*, 147−155.
(32) Jones, B. L.; Budde, A. D. Endoproteinases and the hydrolysis of malt proteins during mashing. In *European Brewery Convention Proceedings of the 27th Congress, Cannes, France*; Secretariat of EBC: Leiden, The Netherlands, 1999; pp 611−618.
(33) Sundblom, N.-O.; Mikola, J. On the nature of the proteinases secreted by the aleurone layer of barley grain. *Physiol. Plant.* **1972**, *27*, 281−284.

Received for review April 2, 2001. Revised manuscript received July 26, 2001. Accepted August 15, 2001. We are grateful to the American Malting Barley Association and Anheuser-Busch, Inc., for supplying grants that paid for part of this research. 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 a name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

JF0104331