

Proteolytic Enzymes in Germinating Rye Grains

Kristof Brijs,^{1,2} Isabel Trogh,¹ Berne L. Jones,³ and Jan A. Delcour¹

ABSTRACT

Cereal Chem. 79(3):423–428

The proteolytic activities during rye (*Secale cereale* L. 'Humbolt') grain germination were monitored using in-solution methods and one- and two-dimensional PAGE with gels that contained incorporated substrate proteins. The total proteolytic activity increased during the first three days of germination, but not after that. The proteinase activity was measured at pH 3.8, 6.0, and 8.0 in the presence and absence of class-specific pro-

teinase inhibitors. This indicated that enzymes from all four proteinase classes were present during the germination process. Germinated rye grain contained mainly aspartic and cysteine proteinase activities that are especially active at pH 3.8. Serine- and metallo-proteinases were less abundant. Overall, the pattern of hydrolysis was very similar to that observed during barley and wheat germination.

The life cycle of a cereal grain has two phases, development and germination, that are separated by a period of dormancy (Rock and Quatrano 1995). During seed germination, much of the amino acid supply necessary for the growth of the emerging seedling comes from the degradation of seed storage proteins. The latter are synthesized during seed maturation and deposited in the protein bodies, which are specialized vacuoles. The mobilization of the seed storage proteins takes place in a sequential manner (Enari and Sopanen 1986) by specific proteases that are synthesized de novo and transported into the protein bodies. There are conflicting views about which proteases are responsible for the beginning of hydrolysis (Rastogi and Oaks 1986; Shutov and Vaintraub 1987; Dunaevsky et al 1989). Proteases are not only important for storage protein hydrolysis, they are also responsible for turning over proteins, post-translational modifications, the activation and inactivation of enzymes, and plant defense.

Little is known about the proteolytic enzymes in germinated rye. In contrast, the proteases that appear during the germination of barley (Wrobel and Jones 1992a,b; Zhang and Jones 1995a,b) and wheat (Dominguez and Cejudo 1995) are well studied. Using a non-denaturing electrophoretic system with gel-incorporated protein substrate, Wrobel and Jones (1992a) detected seven proteolytic bands in barley green malt. Five of these bands were maximally active at pH 3.8. Up to 20 proteolytic bands could be detected in extracts from germinated wheat grains (Dominguez and Cejudo 1995). It is clear from several reports that total proteolytic activity increases during germination. Wrobel and Jones (1992b) investigated the temporal pattern of appearance of the proteinases during germination, the sensitivities to class-specific inhibitors, and where the proteinases reside. Two-dimensional (2-D) gel electrophoresis (IEF × PAGE) with incorporated substrates separated 42 activities in germinating barley grain, which fell into five groups on the basis of pI values, PAGE mobilities, and biochemical characteristics (Zhang and Jones 1995a). About two-thirds of these enzymes were cysteine proteinases. The barley cysteine proteinases apparently hydrolyze the bulk of the hordeins, which are the major barley endosperm storage proteins. The purification and characterization of some of these cysteine proteinases has been described in a number of reports (Koehler and Ho 1988; Poulle and Jones 1988; Phillips and Wallace 1989; Zhang and Jones 1996).

The aim of this study was to initiate the characterization of malted rye proteinases by using two different methods to analyze their activities. We used a qualitative 2-D (IEF × PAGE) method to measure the

heterogeneity of the proteases and an in-solution method with different substrates to quantitatively measure the activities of the different proteinase groups.

MATERIALS AND METHODS

Materials

The rye cultivar Humbolt was from AVEVE (Landen, Belgium). All reagents were purchased from Sigma-Aldrich (Bornem, Belgium) and were analytical grade unless otherwise specified. Electrophoresis media and molecular weight markers were from Pharmacia Biotech (Uppsala, Sweden).

Seed Germination

Rye grains were germinated on a pilot-scale system (Joe White Malting Systems, Melbourne, Australia) under optimal conditions of temperature, humidity, and aeration. The grains were steeped (soaked in water with alternating 4-hr air rests) at 18°C to 45% moisture (16 hr) and germinated at 18°C for five days. During germination, the seeds were slowly rotated in the dark at 100% humidity. During this process, grain samples were collected before steeping (ungerminated or resting seeds), at the end of steeping (out of steep), and every 24 hr thereafter for five more days. The collected samples were immediately frozen in liquid nitrogen and stored at -20°C. Before use, the rye samples were freeze-dried.

The rye samples were not kilned after germination to avoid any loss of activity due to high kilning temperatures. Earlier qualitative 2-D (IEF × PAGE) analyses, which allowed the study of individual proteases, showed that some of barley green malt proteases were affected by heating at 68 and 85°C during the final stages of kilning. However, these changes obviously did not decrease the overall proteolytic activity (Jones et al 2000).

Rye Milling

Germinated rye milling fractions were obtained by milling three-day germinated and freeze-dried Humbolt samples (5.0 kg) at 14.5% moisture on a MLU-202 laboratory mill (Bühler, Uzwil, Switzerland) according to Approved Method 26-31 (AACC 2000) to yield eight streams: bran, shorts, and six flour fractions (B1, B2, B3, C1, C2, and C3). B fractions are from successive break rolls and C fractions are from successive reduction rolls (Delcour et al 1989). The ash and moisture contents of the different milling fractions were measured using Approved Methods 08-01 and 44-19, respectively (AACC 2000). Protein contents (dry basis, N × 5.7) were determined by a micro Kjeldahl procedure (Jones 1991).

Extraction of Proteolytic Enzymes

Germinated rye whole meal or milling fractions were extracted by mechanical shaking with 0.05M sodium acetate buffers (pH 5.0) containing 2.0 mM cysteine (1:10, w/v) for 30 min at 7°C. After centrifugation (15,000 × g, 4°C, 15 min), the supernatants were paper-filtered and germinated rye whole meal extracts (GRWME)

¹ Laboratory of Food Chemistry, Katholieke Universiteit Leuven, Kasteelpark Arenberg 20, B-3001 Leuven, Belgium.

² Corresponding author. Phone: (+32)-16-321582. Fax: (+32)-16-321997. E-mail: kristof.brijs@agr.kuleuven.ac.be

³ USDA-Agricultural Research Service, Cereal Crops Research Unit, 501 N. Walnut St., Madison, WI 53705.

or germinated rye extracts (GRE) from different milling fractions were used to analyze proteolytic activities. In some instances, grains were bleach-treated with 20% sodium hypochlorite to kill or wash away contaminating microbes.

Proteolytic Activities

Hemoglobin hydrolyzing activities. Hemoglobin solution (0.25 mL, 1.0%, w/v, in 0.2M sodium acetate buffer, pH 4.0), 0.2 mL of sodium acetate buffer (0.2M, pH 4.0), and 0.05 mL of GRWME or GRE were mixed. After incubation (150 min, 40°C), the reaction was stopped by the addition of 0.4 mL of cold (7°C) 10.0%, w/v, trichloroacetic acid (TCA); precipitated proteins were removed by centrifugation (10,000 × g, 10 min). The free α-amino nitrogen levels of the supernatants were assayed with trinitrobenzene-sulfonic acid (TNBS) reagent (0.3%, v/v, in 0.2M sodium phosphate buffer, pH 8.0) using L-leucine as standard. To this end, the supernatant (0.025 mL) and TNBS reagent (0.225 mL) were incubated for 20 min at

50°C, after which the reaction was stopped with 0.2M HCl (0.75 mL). The absorbance of the solution at 340 nm was measured. Under such conditions, the absorbance versus time plot was linear for at least 210 min of incubation. One unit of activity corresponds to the enzyme activity that liberated 1 mg of L-leucine/hr under the assay conditions. For the following discussion, hemoglobin hydrolyzing activity is considered to be a measure of both endo- and exoproteolytic activity.

Azocasein hydrolyzing activities. The endoproteolytic activities were measured using azocasein as substrate. The standard reaction mixture was prepared by mixing 0.35 mL of azocasein (1.4%, w/v, in 0.05M McIlvaine buffer, pH 5.5) and 0.25 mL of GRWME or GRE. After incubation (4 hr, 40°C), the reaction was stopped by the addition of 0.5 mL of cold (7°C) 10% TCA; precipitated proteins were removed by centrifugation (10,000 × g, 10 min). Dilute sodium hydroxide (0.5M) was added to an equal volume of supernatant. The mixture was allowed to stand for 20 min and absorbance

TABLE I
Changes in Total Proteolytic (Hemoglobin), Endoproteolytic (Azocasein), Carboxypeptidase (CPA), Aminopeptidase (LPA), and Nα-Benzoyl-p-Nitroanilide (BAPA) Hydrolase Activities in Rye During Germination^a

Rye Sample	Hemoglobin ^b	Azocasein ^c	LPA ^d	BAPA ^e	CPA ^b
Ungerminated	3.91	1.40	1,769	1,313	11.88
After steeping	6.81	2.96	2,214	1,319	18.09
Day 1	20.55	6.70	3,689	1,365	22.79
Day 2	25.17	8.04	3,722	1,425	25.08
Day 3	29.54	8.90	3,590	1,480	24.92
Day 4	27.38	8.64	3,775	1,557	25.12
Day 5	29.12	9.80	4,042	1,469	27.22

^a Proteinases extracted with 0.05M sodium acetate buffer (pH 5.0); activities are expressed as U/g of rye whole meal.

^b One unit of activity (U) corresponds to liberation of 1 mg of L-leucine/hr at pH 4.0 and 40°C under the assay conditions specified.

^c Activity reported as increase in absorbance at 440 nm/hr at pH 5.5 and 40°C under the assay conditions specified.

^d One unit of activity (U) corresponds to the liberation of 1 μmol of p-nitroaniline/hr at pH 7.2 and at 30°C under the assay conditions specified.

^e One unit of activity (U) corresponds to the liberation of 1 μmol of p-nitroaniline/hr at pH 8.6 and at 30°C under the assay conditions specified.

TABLE II
Laboratory Milling Results^a for Humbolt Rye Germinated for Three Days and Proteolytic Activities in Milling Fractions

Fractions ^b	Yield (%)	Nitrogen Content (%)	Ash Content (%)	Hydrolyzing Activity (U/g of milled fraction)	
				Hemoglobin ^c	Azocasein ^d
Whole kernel	100.0	2.31	2.18	23.47	4.22
B1	12.4	1.46	0.90	14.88	2.29
B2	5.7	1.80	1.12	13.36	2.21
B3	2.3	2.00	1.23	12.74	2.02
C1	36.8	1.79	1.11	15.26	1.85
C2	6.6	2.48	1.66	12.59	2.09
C3	2.8	2.36	1.59	9.73	1.37
Shorts	24.5	3.43	4.00	28.81	5.22
Bran	8.2	2.79	5.26	52.47	9.57

^a All results % of dry matter.

^b B fractions from successive break rolls and C fractions from successive reduction rolls.

^c One unit of activity (U) corresponds to the liberation of 1 mg of L-leucine/hr at pH 4.0 and 40°C under the assay conditions specified.

^d Activity reported as increase in absorbance at 440 nm/hr at pH 5.5 and at 40°C under the assay conditions specified.

TABLE III
Effect of Different Synthetic Inhibitors on Proteolytic Activity of Three-Day Germinated Humbolt Rye Grain and Bran Fraction Using Hemoglobin and Azocasein as Substrate Proteins

Inhibitor	Protease Classes Inhibited	Concentration (mM)	% Inhibition Against Hemoglobin		% Inhibition Against Azocasein	
			Grain	Bran	Grain	Bran
None			0	0	0	0
Ethanol		2%	1	1	1	1
o-Phenanthroline ^a	Metallo	5.0	14	1	22	10
Pepstatin A ^a	Aspartic	0.1	19	25	50	54
PMSF ^a	Serine	2.0	35	30	15	10
TLCK	Serine (trypsin)	1.0	64	40	12	35
TPCK ^a	Serine (chymotrypsin)	1.0	39	26	20	28
Leupeptin	Cysteine (some serine)	0.02	59	41	27	36
NEMI ^a	Cysteine	2.0	37	37	50	31
Iodoacetamide	Cysteine	1.0	28	46	17	29
E-64	Cysteine	0.05	59	55	40	47

^a Inhibitors were dissolved in ethanol such that the ethanol concentration in the final assay was 2%.

at 440 nm was measured. The activities were expressed as the increase in absorbance at 440 nm/hr under the conditions of the assay that yielded linear absorbance versus time plots.

Carboxypeptidase activity. *N*-Carbobenzoxy-L-phenylalanyl-L-alanine (CPA) was used for detecting the carboxypeptidase activity. GRWME or GRE (0.5 mL) was mixed with 0.5 mL of the substrate solution (2.0 mM CPA in 0.05M sodium acetate buffer, pH 4.0). The mixture was incubated for 1 hr at 40°C. The reaction was stopped by the addition of 0.5 mL of ninhydrin solution (Weegels et al 1992) (1:1:1, v/v, mixture of 2.0M citric acid and sodium citrate buffer, pH 5.0; 2%, w/v, ninhydrin in 1:1, v/v, deionized water and 2-methoxyethanol; 8.4 mM tin chloride in deionized water). The mixture was incubated for 20 min at 100°C. L-Leucine was used as a standard. After centrifugation (10,000 × g, 10 min), the supernatant was diluted appropriately in 1:1, v/v, deionized water and 2-methoxyethanol, and the absorbance at 570 nm was measured. Under these assay conditions, the release of L-leucine versus time was linear. One unit of activity corresponds to the liberation of 1 mg of L-leucine/hr at pH 4.0 and 40°C. In these three assays, the activities were corrected for control values by incubating the substrates and enzyme solutions separately for the appropriate time, combining them and immediately stopping the reactions as described for the respective assays.

Aminopeptidase and BAPA-ase activities. L-Leucine-*p*-nitroanilide (LPA) and *N*- α -benzoylarginine-*p*-nitroanilide (BAPA, Janssen Chimica, Belgium) were used as substrates for the aminopeptidase and BAPA-ase activities, respectively. The activities were determined by mixing 0.2 mL of GRWME or GRE, 0.8 mL buffer (0.2M McIlvaine buffer, pH 7.2, or 0.2M Tris-HCl buffer, pH 8.6, for LPA or BAPA

respectively) and 0.2 mL of substrate solution (0.1 g of LPA or BAPA in 2.5 mL of dimethylsulphoxide and diluted to 250.0 mL with the appropriate buffer). Enzyme reactions were monitored colorimetrically at 410 nm and conducted at 30°C. One unit of activity corresponds to the (linear) release of 1 μ mol of *p*-nitroaniline/hr from the relevant substrate at pH 7.2 (LPA) or pH 8.6 (BAPA). In all enzyme assays, the results are mean values of triplo-measurements and the experimental error is <2% (defined as the difference between individual and mean values).

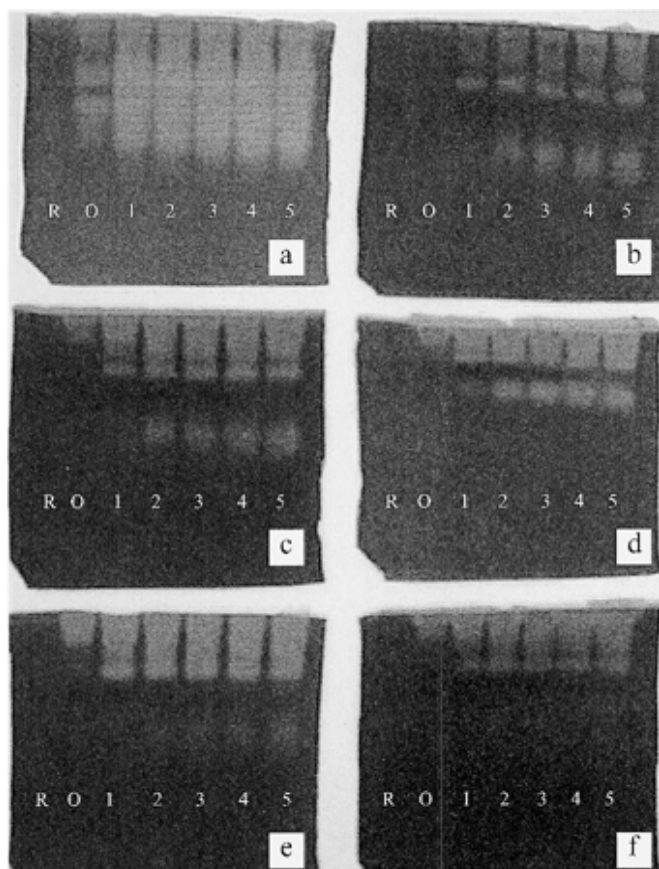


Fig. 1. PAGE showing proteolytic activity of rye extracts prepared from grain at different germination stages (R, resting seeds; O, out of steep; 1, 2, 3, 4, 5, days of germination). Gels contained incorporated gelatin (0.1%) incubated overnight in 0.1M sodium acetate buffer (pH 3.8) (**a and b**), 0.1M citrate phosphate buffer (pH 6.0) (**c and d**), and 0.1M Tris-HCl (pH 8.0) (**e and f**). All incubation buffers contained 2.0 mM cysteine. Gels were incubated in the absence (a, c, e) or presence (b, d, f) of 1.0 mM E-64.

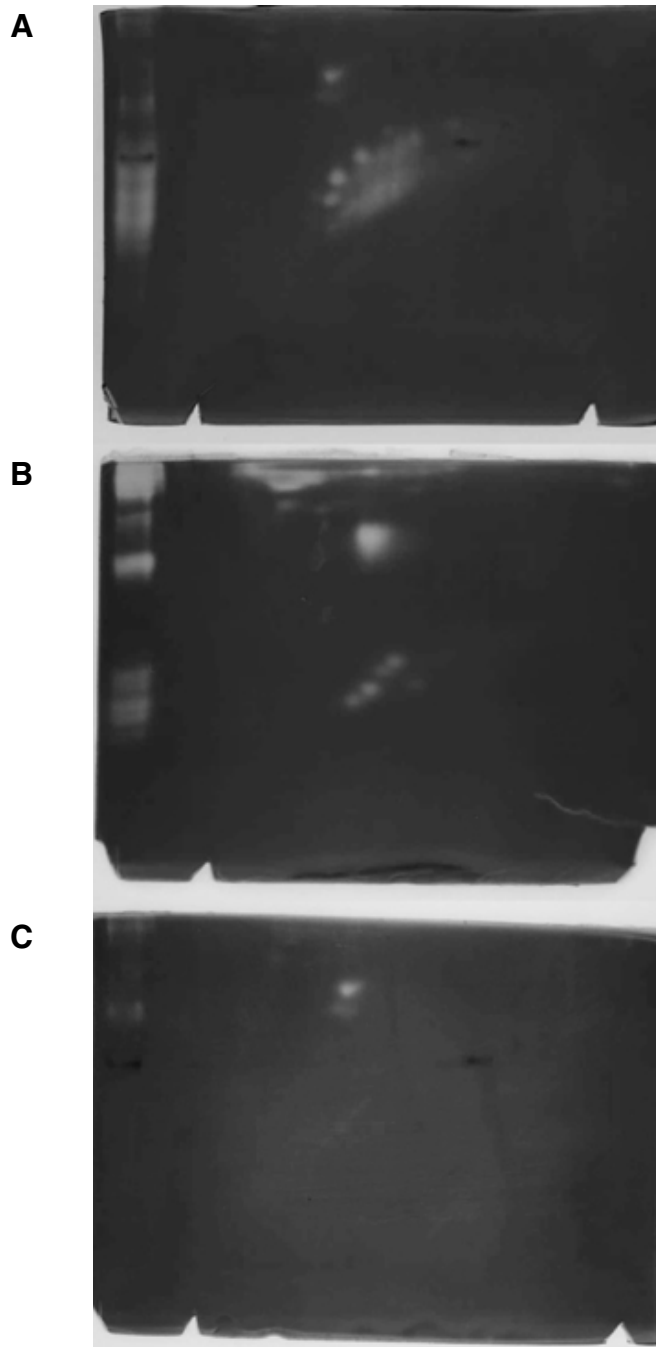


Fig. 2. 2-D (IEF × PAGE) separation of germinated rye endoproteases. PAGE gels contained incorporated gelatin. After proteinases were separated, gels were incubated with 100 mM buffer containing 2.0 mM cysteine at **A**, pH 3.8 (sodium acetate); **B**, pH 6.0 (citrate phosphate); and **C**, pH 8.0 (Tris-HCl). The *x*-axis indicates pI of each activity; *y*-axis indicates distance moved during PAGE. Left side of each gel shows a separation due to PAGE only.

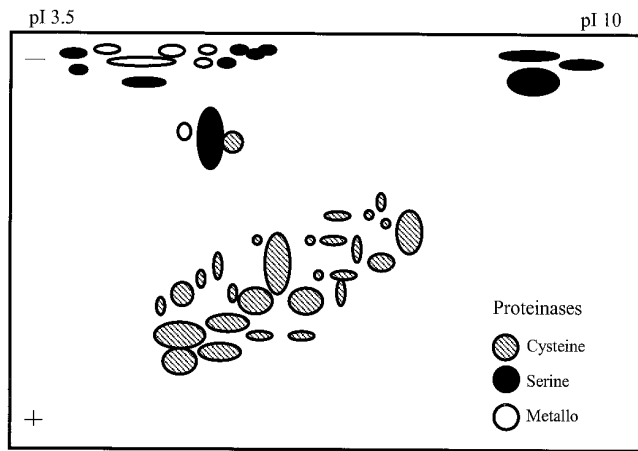


Fig. 3. Composite diagram of germinated rye proteolytic activities detected with gels that contained incorporated gelatin after separation of proteinases by 2-D electrophoresis. The x-axis indicates pI of each activity; y-axis indicates distance moved during PAGE.

Protein

The protein concentrations of the GRWME or GRE solutions were determined using the Coomassie brilliant blue method (Bradford 1976) with bovine serum albumin as standard.

pH and Temperature

The pH optima for the hydrolysis of hemoglobin and azocasein were determined at 40°C with 0.2M sodium acetate buffers at pH 3.0–6.0 and 0.05M McIlvaine buffers at pH 5.0–6.5. The temperature optimum for the hydrolysis of hemoglobin was determined with 0.2M sodium acetate buffer (pH 3.5) at 22–70°C.

Electrophoresis and Endoproteolytic Activities in Gels

The 2-D (IEF × PAGE) method for separating and detecting proteinases was conducted as described by Zhang and Jones (1995a). After IEF, the proteinases were separated in 2-D PAGE in a non-denaturing 11% acrylamide gel that contained 0.1% immobilized substrate. When the IEF gel was incorporated in 2-D PAGE gel, a single aliquot of the extract was applied to an adjacent well. After PAGE, the gels were incubated overnight at 45°C in 0.1M sodium acetate buffer (pH 3.8), 0.1M citrate-phosphate buffer (pH 6.0), or 0.1M Tris-HCl buffer (pH 8.0). Two mM cysteine was added to all incubation buffers. The gels were stained with 0.1% amino black, destained with 30:10:60 (v/v) methanol and acetic acid water, and stored in the destaining solution. The method stained the substrate protein, leaving clear areas where the endoproteases had hydrolyzed the substrate. In 1-D electrophoresis, the samples were loaded into wells in the PAGE slab gels.

Proteinase Inhibitor Analyses

In-solution assays. The effects of different inhibitors on the hemoglobin and azocasein hydrolyzing activities were evaluated at 45°C with hemoglobin in 0.2M sodium acetate buffer (pH 4.0) and azocasein in 0.05M McIlvaine buffer (pH 5.5). Inhibitors used were tetrasodium EDTA (5.0 mM, UCB, Belgium), *N*-ethylmaleimide (NEMI, 2.0 mM), leupeptin (0.02 mM), iodoacetamide (1.0 mM), phenylmethylsulphonyl fluoride (PMSF, 2.0 mM), *N*- α -*p*-tosyl-L-lysine chloromethyl ketone (TLCK, 1.0 mM), *N*- α -*p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK, 1.0 mM), *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64, 0.05 mM), and pepstatin A (0.1 mM). NEMI, TPCK, PMSF, and pepstatin A were dissolved in ethanol such that there was 2% ethanol in the final assay.

Gel Electrophoresis Assays

The gels were developed as described, except that inhibitors were added to the buffers to give final concentrations of E-64 (10.0 μ M),

pepstatin A (20.0 μ M), PMSF (10.0 mM), and *o*-phenanthroline (1.0 mM).

RESULTS AND DISCUSSION

Endoproteolytic, exoproteolytic, carboxypeptidase, aminopeptidase, and *N*- α -benzoyl-arginine-*p*-nitroanilide hydrolyzing activities were all detected in 0.05M sodium acetate (pH 5.0) extracts of whole meal prepared from ungerminated Humbolt rye (Brijs et al 1999).

Characteristics of Whole Meal Rye Extracts During Germination

Sodium acetate buffer (0.05M, pH 5.0) is an efficient extraction buffer for solubilizing the proteolytic enzymes in ungerminated rye whole meal (Brijs et al 1999). The addition of 2.0 mM cysteine to this buffer strongly increased the extracted proteolytic activity from germinated rye. Therefore, it was added to all of the extraction buffers. Table I shows that the overall rye proteolytic activity increased during the first three days of germination and remained constant after that. The hemoglobin hydrolyzing activity after three days of germination was $\approx 7.5\times$ higher than that of ungerminated rye grains. Similar results were found when the proteolytic activity in germinated rye grain samples was measured with azocasein (Table I). When the exoproteolytic activities were measured with CPA or LPA as substrates, the increase of activity was not as pronounced as with hemoglobin and azocasein. However, there was still more exoproteolytic activity in the three-day germinated grains than in the ungerminated grains. Conversely, the BAPA-ase activity remained almost constant during the germination process. To ensure that the activities detected were not due to microbial contamination, bleach-treated samples were analyzed for proteinase activity as well. No diminution in protease activity was found. These results are in line with those of Wrobel and Jones (1992b), who found that the husks of five-day germinated barley grains showed no proteinase activity and that bleach-treating the germinated grains did not result in any loss of proteinase bands relative to those of the untreated germinated barley.

The pH and temperature optima of the hemoglobin hydrolyzing activity of three-day germinated Humbolt extract were ≈ 3.5 and 45–50°C, respectively (results not shown). These results are comparable with those reported earlier for ungerminated rye grains (Brijs et al 1999).

The increase in proteolytic activity during germination was also monitored using electrophoresis with gels that contained incorporated gelatin. Three identical gels were run and the proteolytic activities of each were developed by incubation at a different pH-value. The results are shown in Fig. 1. Under the most acidic conditions tested (pH 3.8), considerable activity was already present in the grains after steeping (Fig. 1a). These activities were enhanced after the first day of germination. Activities after additional germination days were very similar. The proteinase activities at pH 6.0 (Fig. 1c) and 8.0 (Fig. 1e) were almost identical to each other at each germination time, although the activity bands were more intense at pH 8.0 than at pH 6.0. The number and intensities of the bands increased until the third day of germination. The patterns of the three-, four-, and five-day samples were almost identical. However, it was sometimes difficult to detect individual bands under these assay conditions (45°C, overnight) due to the high activities. It was easier to distinguish the individual bands when shorter incubation times (2.5 hr) were used for the four- and five-day germinated grains (results not shown).

To evaluate the impact of the cysteine proteinases during germination, E-64 was added to the incubation buffers to inhibit their activities. At pH 3.8 (Fig. 1b), cysteine proteinases were responsible for most of the proteolytic activity. Most of the activity bands disappeared in the presence of this inhibitor. The remaining hydrolysis spots were from serine- and metallo-proteinases, as evidenced by their inhibition with PMSF and *o*-phenanthroline

(results not shown). At pH 6.0 (Fig. 1d) and pH 8.0 (Fig. 1f), the effects of E-64 were decreased, although some activity bands disappeared. These results indicate that some cysteine proteinases are active at neutral and higher pH values. These results are in accordance with earlier results found with germinated barley (Wrobel and Jones 1992a) and wheat (Dominguez and Cejudo 1995) proteinases.

Rye Milling

Data on the laboratory milling of three-day germinated Humbolt rye are listed in Table II. The flour yield was 67%, which was significantly higher than that of ungerminated rye grains. This is probably due to an increased degradation of the inner grain cell wall components.

Assays of the milling fractions indicated that the proteolytic activities increased steadily as the nitrogen and ash contents of the fractions increased. The hemoglobin and azocasein hydrolyzing activities in the bran and the shorts were higher than those of the different flour fractions and GRWME. The bran fraction had the highest proteolytic activity, but the high activity of the C1 fraction, compared with that of the ungerminated rye sample, is remarkable. The relative amount of the C1 milling fraction of the germinated rye increased, while the bran and shorts fractions decreased. The proteolytic activities of the different milling fractions of the germinated rye grains were much higher than those from the ungerminated rye grains (Brijs et al 1999).

Effects of Inhibitors on Proteolytic Enzymes in Germinated Rye Grain

The influences of different synthetic protease inhibitors on the proteolytic activities of GRWME as well as bran extract were tested (Table III). All four classes of proteases were present, as all inhibitors decreased the total activity in both the GRWME and bran extract. *o*-Phenanthroline, a potential inhibitor of metallo-proteases, had little effect on the total proteolytic activities. In contrast, pepstatin A inhibited 50 and 54%, respectively, of the azocasein hydrolyzing activity present in GRWME and bran extracts. With hemoglobin as substrate, the inhibition effects were smaller (only 19 and 25%). After the germination of rye grains, there were still aspartic proteinases present. During the germination of barley grains, the activities of the aspartic proteinases increased, although six aspartic proteinase activities were found in resting barley seeds, while only four activities were detected in green malt (Zhang and Jones 1999). The serine-proteases that were inhibited by PMSF were mostly exoproteolytic, as the inhibition of the hydrolysis of azocasein was very low. This was not the case with hemoglobin. Based on the inhibition effects of TLCK and TPCK, we can conclude that most of the serine proteinases were trypsin-like. A great part of the proteolytic activity in the GRWME was caused by cysteine-proteinases. Although leupeptin, NEMI, iodoacetamide, and E-64 are all cysteine proteinase inhibitors, they reacted in different ways, resulting in different extents of inhibition of the proteolytic activity. Based on these results (Table III), it is clear that the cysteine proteinases are not the major class of proteinases in three-day germinated rye grain. Addition of pepstatin A in the azocasein assay and TLCK in the hemoglobin assay gives as much inhibition as any of the cysteine proteinase inhibitors.

Analysis of Proteolytic Activities Using 2-D IEF \times PAGE

When the proteinases of malted rye grains were separated using 2-D IEF \times PAGE and detected by the hydrolysis of gelatin incorporated into the PAGE gels, multiple proteinases were observed (Fig. 2). Large hydrolysis spots were detected by incubating the gel at pH 3.8 (Fig. 2A). These spots disappeared completely in the presence of E-64 (results not shown). This indicates that germinated rye grains contained high levels of cysteine proteinases. At pH 6.0 (Fig. 2B), gelatin hydrolysis was mainly due to serine- and metallo-proteinases. These enzymes migrate near the top of the gel.

A large hydrolysis zone that was detectable at pH 6.0 disappeared completely in the presence of PMSF (results not shown). All the hydrolysis spots that were detected are summarized in a composite diagram in Fig. 3. Gels were made at pH 3.8, 6.0, and 8.0. Clear hydrolysis spots were detected and marked as circles in the composite diagram. Later, the spots were characterized by incubating the gels under the same conditions using class-specific inhibitors.

When judged by this technique, the differences between germinated barley (Zhang and Jones 1995a) and germinated rye proteinases are small. The main difference was a greater relative level of metallo-proteinases in germinated rye. However, at pH 8.0, the metallo-proteinases in germinated barley are still very active, whereas those in germinated rye were not. There were also rather major differences in the level and localization of the cysteine proteinases. Furthermore, there were about five hydrolysis spots visible after incubation of the gel at pH 6.0 and slightly visible at pH 8.0. Studies with class-specific inhibitors showed that these were cysteine proteinases active at higher pH values. The results with germinated oats (Mikola and Jones 2000) were different. Most of the proteinases in germinated oat are serine- and metallo-proteinases. No aspartic or cysteine proteinases were detected. However, the in-solution analyses of oat proteases gave somewhat different results from those obtained with 2-D IEF \times PAGE. In the presence of calcium and cysteine, one-third of the in-solution activity was due to cysteine proteinases. Different results may simply reflect the fact that the enzymes hydrolyze the substrate differently, depending on whether it is dissolved or insoluble.

CONCLUSIONS

The rye cultivar Humbolt was selected because previous studies (Brijs et al 1999) had shown that this cultivar yielded high proteolytic activities. It was germinated at optimal conditions of temperature, aeration, and humidity. Proteolytic activities were monitored using in-solution methods and 1-D and 2-D PAGE with incorporated substrates. The sodium acetate buffer (0.05M, pH 5.0) containing 2.0 mM cysteine was used to extract the proteolytic enzymes. When hemoglobin and azocasein were used as substrates, the overall proteolytic activity was significantly higher after steeping. Furthermore, the activity increased during the first three days of germination but not after that. The hemoglobin hydrolyzing activity of extracts from three-day germinated rye was maximal at pH \approx 3.5 and 45–50°C. After milling, large levels of proteolytic activity occurred in the C1 flour fraction and less activity was found in the bran and shorts.

The use of class-specific inhibitors showed that the four classes of proteinases were present. In whole meal, pepstatin A inhibited 50% of the proteolytic activity with azocasein as substrate. With hemoglobin, only 19% of the activity was inhibited under these conditions. It was clear that there were still aspartic proteinases present in three-day germinated rye grain. Cysteine proteinases are mainly located in the starchy endosperm. Based on our results for proteolytic enzymes in germinated rye grains, it appears that the hydrolysis of storage proteins in rye during germination is probably due to cysteine and aspartic proteinases.

ACKNOWLEDGMENTS

K. Brijs acknowledges the receipt of a scholarship from the Instituut voor de aanmoediging van Innovatie door Wetenschap en Technologie in Vlaanderen (Brussels, Belgium). The fund for Scientific Research (Flanders, Belgium) (FWO) is thanked for receipt of a travel grant. We gratefully acknowledge Laurie Marinac for excellent technical assistance.

LITERATURE CITED

- American Association of Cereal Chemists. 2000. Approved Methods of the AACC, 10th ed. Methods 08-01, 26-31, and 44-19, The Association: St. Paul, MN.
- Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principles of protein-

- dye binding. *Anal. Biochem.* 72:248-254.
- Brijs, K., Bleukx, W., and Delcour, J. A. 1999. Proteolytic activities in dormant rye (*Secale cereale* L.) grain. *J. Agric. Food Chem.* 47:3572-3578.
- Delcour, J. A., Vanhamel, S., and De Geest, C. 1989. Physicochemical and functional properties of rye nonstarch polysaccharides. I. Colorimetric analysis of pentosans and their relative monosaccharide compositions in fractionated (milled) rye products. *Cereal Chem.* 66:107-111.
- Dominguez, F., and Cejudo, F. J. 1995. Pattern of endoproteolysis following wheat grain germination. *Physiol. Plant.* 95:253-259.
- Dunaevsky, Y. E., Sarbakanova, S. T., and Belozersky, M. A. 1989. Wheat carboxypeptidase and joint action on gliadin of proteases from dry and germinating seeds. *J. Exp. Bot.* 40:1323-1329.
- Enari, T.-M., and Sopanen, T. 1986. Mobilisation of endospermal reserves during the germination of barley. *J. Inst. Brew.* 92:25-31.
- Jones, J. B., ed. 1991. Kjeldahl Method for Nitrogen (N) Determination. Micro-Macro Publishing: Athens, GA.
- Jones, B. L., Marinac, L. A., and Fontanini, D. 2000. Quantitative study of the formation of endoproteolytic activities during malting and their stabilities to kilning. *J. Agric. Food Chem.* 48:3898-3905.
- Koehler, S., and Ho, T.-H. 1988. Purification and characterization of gibberellic acid-induced cysteine endoproteases in barley aleurone layers. *Plant Physiol.* 87:95-103.
- Mikola, M., and Jones, B. L. 2000. Electrophoretic and "in solution" analysis of endoproteases extracted from germinated oats. *J. Cereal Sci.* 31:15-23.
- Phillips, H. A., and Wallace, W. 1989. Cysteine endopeptidase from barley malt which degrades hordein. *Phytochemistry* 28:3285-3290.
- Pouille, M., and Jones, B. L. 1988. A proteinase from germinating barley. I. Purification and some physical properties of a 30 kDa cysteine endoprotease from green malt. *Plant Physiol.* 88:1454-1460.
- Rastogi, V., and Oaks, A. 1986. Hydrolysis of storage proteins in barley endosperms: Analysis of soluble products. *Plant Physiol.* 81:901-906.
- Rock, C. D., and Quatrano, R. 1995. The role of hormones during seed development. Pages 671-697 in: *Plant Hormones, Physiology, Biochemistry and Molecular Biology*, 2nd Ed. P. J. Davies, ed. Kluwer Academic: Dordrecht, The Netherlands.
- Shutov, A. D., and Vaintraub, I. A. 1987. Degradation of storage proteins in germinating seeds. *Phytochemistry* 26:1557-1566.
- Weegels, P. L., Marseille, J. P., and Hamer, R. J. 1992. Enzymes as a processing aid into the separation of wheat flour in starch and gluten. *Starch* 44:44-48.
- Wrobel, R., and Jones, B. L. 1992a. Electrophoretic study of substrate and pH dependence of endoproteolytic enzymes in green malt. *J. Inst. Brew.* 98:471-478.
- Wrobel, R., and Jones, B. L. 1992b. Appearance of endoproteolytic enzymes during the germination of barley. *Plant Physiol.* 100:1508-1516.
- Zhang, N., and Jones, B. L. 1995a. Characterization of germinated barley endoproteolytic enzymes by two-dimensional gel electrophoresis. *J. Cereal Sci.* 21:145-153.
- Zhang, N., and Jones, B. L. 1995b. Development of proteolytic activities during barley malting and their localization in the green malt kernel. *J. Cereal Sci.* 22:147-155.
- Zhang, N., and Jones, B. L. 1996. Purification and partial characterization of a 31-kDa cysteine endopeptidase from germinated barley. *Planta* 199:565-572.
- Zhang, N., and Jones, B. L. 1999. Polymorphism of aspartic proteinases in resting and germinating barley seeds. *Cereal Chem.* 76:134-138.

[Received May 21, 2001. Accepted January 14, 2002.]