Proteolytic Activities in Dormant Rye (*Secale cereale* L.) Grain

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Endoproteolytic, exoproteolytic, carboxypeptidase, aminopeptidase, and *N*-α-benzoyl-arginine-*p*-nitroanilide hydrolyzing activities were detected in 0.05 M sodium acetate buffer (pH 5.0) extracts of whole meal of the rye (*Secale cereale* L.) varieties Amando, Halo, and Humbolt. The proteolytic enzymes of Humbolt, the variety with the highest proteolytic activity, optimally hydrolyzed hemoglobin around pH 3.5 and 40–45 °C. In the different milling fractions of Humbolt, azocasein and hemoglobin hydrolytic activities were especially found in the bran and shorts. Proteolytic enzymes in the bran extract were concentrated in the 35–60% ammonium sulfate precipitate. Pepstatin A, an inhibitor of aspartic proteases, reduced ~88 and ~75% of the hemoglobin and azocasein hydrolyzing activities of this precipitate, respectively. Phenylmethanesulfonyl fluoride, an inhibitor of serine proteases, inhibited ~33% of both cited activities. Both rye and wheat storage proteins were degraded by Humbolt rye whole meal enzyme extract and the above-mentioned ammonium sulfate rye bran fraction in vitro. With the latter fraction digestion was more pronounced.

Keywords: *Proteolytic enzymes; rye (Secale cereale L.); storage proteins*

INTRODUCTION

Rye (*Secale cereale* L.) is a traditional raw material for bread-making that adds variety to the European bread market. The present world harvest of rye is 30–35 million tonnes, 95% of which is produced in the northern part of the region from the Nordic Sea to the Ural Mountains (Poutanen, 1997).

Despite the recent interest in rye, little is known about its proteolytic enzymes. Reported studies are scarce and mainly aimed at comparing the proteolytic activity of triticale with that of the parent cereals wheat and rye. However, the proteolytic activities of rye flours are significantly higher than those of wheat flours and close to those of triticale flours (Madl and Tsen, 1973; Singh and Katragadda, 1980). Timmermann and Belitz (1993) found higher proteolytic activities in rye than in wheat, especially toward azocasein, a well-known substrate for endoproteolytic activity (Preston et al., 1978). In contrast, the *N*-α-benzoylarginine-*p*-nitroanilide (BAPA) hydrolyzing activity in wheat was higher than that of rye (Breyer and Hertel, 1974). Despite these observations, it is remarkable that an in-depth investigation of the proteolytic enzymes in rye never occurred.

In contrast, the proteases of resting and germinating seeds of wheat (*Triticum aestivum* L.) (Dominguez and Cejudo, 1995, 1996) and barley (*Hordeum vulgare* L.) (Wrobel and Jones, 1992; Zhang and Jones, 1995a,b) are well characterized. Endoproteases as well as exoproteases appear in the cited cereals. During germination, cysteine proteases are secreted into the starchy endosperm and are mainly responsible for the breakdown of the storage proteins present (Shutov and Vaintraub, 1987). The most abundant group in resting seeds are proteases with acid pH optima. They are found in all parts of the kernel and mainly belong to the aspartic class of proteinases (Voigt et al., 1997).

The present work was carried out to determine the presence and distribution of the different classes of proteolytic enzymes in rye. The study will serve as a starting point for the isolation and purification of new (endo)proteolytic enzymes from rye.

MATERIALS AND METHODS

Materials. Rye cultivars Amando, Halo, and Humbolt (AVEVE, Landen, Belgium) were ground into whole meal with a Cyclotec 1093 sample mill (Tecator, Hogänäs, Sweden).

Commercial wheat gluten with 6.6% moisture content (AACC Method 44-19, 1995) and 81.5% (dry basis, N × 5.7) protein content, as determined by a micro-Kjeldahl procedure (Jones, 1991), was from NV Amylum (Aalst, Belgium).

Rye milling fractions were obtained by milling Humbolt rye (5.0 kg) at a moisture level of 14.5% on an MLU-202 laboratory mill (Bühler, Uzwil, Switzerland) according to Approved Method 26-31 (AACC, 1995) 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 from successive reduction rolls (Delcour et al., 1989). The ash and moisture contents of the different milling fractions were estimated according to AACC Methods 08-01 and 44-19, respectively (AACC, 1995). Protein content (dry basis, N × 5.7) was determined according to a micro-Kjeldahl procedure (Jones, 1991).

All reagents were purchased from Sigma-Aldrich (Bornem, Belgium) and were of analytical grade, unless otherwise specified. Electrophoresis media and molecular weight markers were from Pharmacia Biotech (Uppsala, Sweden).

Extraction of Proteolytic Enzymes. Rye whole meal or milling fractions thereof were extracted (1:10 w/v) for 30 min at 7 °C by mechanical shaking. The extraction buffers included water, 0.05 M sodium acetate buffers (pH 4.0 and 5.0), and 0.2 M sodium acetate buffers (pH 4.0 and 5.0). Following centrifugation (15000*g*, 4 °C, 15 min), the supernatants were paper-filtered and the rye whole meal extracts (RWME) or identical extracts from different rye milling fractions were used as samples for the determination of different proteolytic activities.

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Determination of Proteolytic Activities. *Hemoglobin Hydrolyzing Activities [Modification of the Method of Bushuk et al. (1971)].* Hemoglobin solution [0.25 mL, 1% (w/v) in 0.2 M sodium acetate buffer, pH 4.0], 0.2 mL of sodium acetate buffer (0.2 M, pH 4.0), and 0.05 mL of sample were mixed and incubated at 40 °C during 150 min. The reaction was then stopped by the addition of 0.4 mL cold (7 °C) 10% (w/v) trichloroacetic acid (TCA). Precipitated proteins were removed by centrifugation (10000*g*, 10 min). The supernatants were assayed for free α-amino nitrogen with trinitrobenzenesulfonic acid (TNBS) reagent [0.3% (v/v) in 0.2 M sodium phosphate buffer, pH 8.0] with L-leucine as standard. To this end, supernatant (0.025 mL) and TNBS reagent (0.225 mL) were incubated (20 min, 50 °C). The reaction was stopped with 0.2 M HCl (0.75 mL). Absorbance (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 liberates 1 mg of leucine/h under assay conditions. In what follows, hemoglobin hydrolyzing activity is considered to be a measure of both endo- and exoproteolytic activities.

Azocasein Hydrolyzing Activities [Modification of the Method of Preston et al. (1978)]. Endoproteolytic activity was measured using azocasein as substrate. The standard reaction mixture was prepared by mixing 0.35 mL of azocasein [1.4% (w/v) in 0.05 M McIlvaine buffer, pH 5.5] and 0.25 mL of sample; pH 5.5 was chosen because this was the lowest pH value at which the substrate was soluble. After incubation (4 h, 40 °C), the reaction was stopped by the addition of cold (7 °C) 10% TCA (0.5 mL). Precipitated proteins were removed by centrifugation (10000*g*, 10 min). Dilute sodium hydroxide (0.5 M) was added to an equal volume of supernatant. The mixture was allowed to stand for 20 min, and the absorbance (440 nm) was measured. The activity was expressed as the increase in absorbance at 440 nm per hour under the conditions of the assay that yielded linear absorbance versus time plots.

Azocasein and hemoglobin have been previously used in comparable studies determining proteolytic activities in dormant cereals (Timmermann and Belitz, 1993; Dominguez and Cejudo, 1996; Bleukx et al., 1997) and for this reason were also used in the present study.

CPA-ase Activity [Modification of the Method of Umetsu et al. (1981)]. *N*-Carbobenzoxy-L-phenylalanyl-L-alanine (CPA) was used for the detection of the carboxypeptidase activity. Sample (0.5 mL) was mixed with 0.5 mL of substrate solution (2.0 mM CPA in 0.05 M sodium acetate buffer, pH 4.0). The mixture was incubated for 1 h 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/v) mixture of 2.0 M citric acid/sodium citrate buffer pH 5.0; 2% (w/v) ninhydrin in 1:1 (v/v) deionized water/2-methoxyethanol; 8.4 mM tin chloride in deionized water], and the mixture was incubated for 20 min at 100 °C. L-Leucine was used as a standard. Following centrifugation (10000*g*, 10 min), the supernatant was diluted appropriately in 1:1 (v/v) deionized water/2-methoxyethanol, and the absorbance (570 nm) was measured. Under the 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/h at pH 4.0 and 40 °C.

In these three assays, activity was corrected for control values by incubating substrate and enzyme solution separately for the appropriate time, combining them, and proceeding as described in the respective enzyme assays.

LPA-ase and BAPA-ase Activities [Modification of the Method of Kruger (1971)]. L-Leucine-*p*-nitroanilide (LPA) and *N*-α-benzoylarginine-*p*-nitroanilide (BAPA; Janssens Chimica, Belgium) were used as substrates for aminopeptidase and BAPA-ase activities, respectively. Activities were determined by mixing 0.2 mL of sample, 0.8 mL of buffer (0.2 M McIlvaine buffer, pH 7.2, or 0.2 M Tris-HCl buffer, pH 8.6, for LPA or BAPA, respectively) and 0.2 mL of substrate solution (0.1 g in 2.5 mL of dimethyl sulfoxide and diluted to 250.0 mL with the appropriate buffer). The enzyme reaction was monitored colorimetrically (410 nm) at 30 °C. One unit of activity corresponds to the (linear) release of 1 μmol of *p*-nitroaniline released/h from the relevant substrate at 30 °C and pH 7.2 (LPA) or pH 8.6 (BAPA).

Protein Determination. Protein concentrations in samples were determined according to the Coomassie brilliant blue method (Bradford, 1976) with bovine serum albumin as standard.

pH and Temperature Optima. The pH optima for hydrolysis of hemoglobin and azocasein were determined at 40 °C with, respectively, 0.2 M sodium acetate buffers in a pH range of 3.0−6.0 and 0.05 M McIlvaine buffers in a pH range of 5.0−6.5. Substrate was solubilized in these buffers, and samples were added afterward. The temperature optimum for hydrolysis of hemoglobin was determined with 0.2 M sodium acetate buffer (pH 3.5) in a temperature range of 22−70 °C with a reaction period of 150 min as outlined above. The samples were incubated at the different temperatures for 5 min before substrate was added and the reaction was started.

Effects of Proteinase Inhibitors. The effects of different inhibitors on hemoglobin and azocasein hydrolyzing activities were evaluated at 40 °C with 0.2 M sodium acetate buffer (pH 4.0) for hemoglobin and 0.05 M McIlvaine buffer (pH 5.5) for azocasein. Classical inhibitors used were tetrasodium ethylenediamine tetraacetate (5.0 mM; UCB, Belgium), *p*-hydroxymercuribenzoic acid (1.0 mM), *N*-ethylmaleimide (2.0 mM), leupeptin (0.02 mM), iodoacetamide (1.0 mM), phenylmethanesulfonyl fluoride (PMSF; 2.0 mM), *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (1.0 mM), *N*-α-*p*-tosyl-L-phenylalanine chloromethyl ketone (1.0 mM), and pepstatin A (0.1 mM). *N*-Ethylmaleimide, *N*-α-*p*-tosyl-L-phenylalanine chloromethyl ketone, PMSF, and pepstatin A were dissolved in ethanol such that the concentration of ethanol in the final assay was only 2%.

Ammonium Sulfate (AS) Precipitation. Rye bran (Buhler milling fraction, 300.0 g) was dispersed in 3.0 L of sodium acetate buffer (0.05 M, pH 5.0), shaken for 30 min at 7 °C, and centrifuged (10000*g*, 30 min, 4 °C). Solid AS was slowly added to the supernatant to reach 35% saturation. The mixture was stirred for 30 min and left overnight at 4 °C. The precipitate was then separated by centrifugation (10000*g*, 30 min, 4 °C), dissolved in deionized water, dialyzed for 48 h (4 °C), and lyophilized. This material is hereafter referred to as 0−35% AS. The supernatant fraction was adjusted to 60% saturation in a similar manner, yielding the 35−60% AS and 60%+ AS fractions.

Extraction of Secalins. Secalins were extracted as described by Shewry et al. (1983). Rye whole meal (Humbolt, 50.0 g) was pre-extracted with 250 mL of 95% (v/v) ethanol by stirring for 60 min, and the mixture was centrifuged (8000*g*, 15 min, 4 °C). The residue was stirred (60 min, 20 °C) twice with 250 mL of 50% (v/v) propan-1-ol containing 2% (v/v) 2-mercaptoethanol, with centrifugation after each step (8000*g*, 15 min, 4 °C). The supernatants were combined and mixed with 2 volumes of 1.5 M NaCl. The mixture was left overnight at 4 °C, and precipitated proteins were recovered by centrifugation (8000*g*, 15 min, 4 °C), dissolved in 8.0 M urea, dialyzed extensively against deionized water (4 °C), and freeze-dried.

Evaluation of the Effect of RWME and the 35−60% AS Rye Bran Fraction on Secalins and Gluten. Gluten proteins (20.0 mg) and secalins (20.0 mg) were suspended in 0.2 M sodium acetate buffer, pH 4.0 (1.0 mL). In the case of the gluten proteins, the mixture was boiled for 10 min to inactivate the gluten-associated proteolytic enzymes (Bleukx et al., 1997). For secalins, this was not necessary because no autodigestion occurred during incubation. Then, 0.4 mL of RWME or the 35−60% AS bran fraction [1% (w/v) in 0.05 M sodium acetate buffer, pH 5.0] was added to the substrate suspensions, and the mixtures were incubated with continuous stirring for different periods at 40 °C.

Increase of Free α-Amino Nitrogen as a Function of Time. At different times, 0.05 mL of the suspensions was added to 0.02 mL of sulfosalicylic acid (10%) to stop the reaction. After centrifugation (8000*g*, 10 min, 4 °C), supernatants (0.025 mL) were used to determine free α-amino nitrogen with TNBS reagent, as described above for the assay of hemoglobin hydrolysis.

Table 1. Endoproteolytic, Exoproteolytic, *N*-Carbobenzoxy-L-phenylalanyl-L-alanine Hydrolase (CPA-ase), L-Leucine-*p*-nitroanilide (LPA-ase), and *N*-α-Benzoylarginine-*p*-nitroanilide Hydrolase (BAPA-ase) Activities of the Rye Varieties Amando, Halo, and Humbolt

	units/g of rye[a]		
	Amando	Halo	Humbolt
hemoglobin hydrolyzing activity[b]	3.53	2.86	3.91
azocasein hydrolyzing activity[c]	0.99	1.21	1.40
CPA-ase activity[b]	8.93	10.18	11.88
LPA-ase activity[d]	1788	1716	1769
BAPA-ase activity[e]	948	1608	1313

[a] Proteolytic enzymes were extracted from the three rye varieties with 0.05 M sodium acetate buffer, pH 5.0. [b] One unit of activity corresponds to the liberation of 1 mg of leucine/h at pH 4.0 and 40 °C under the assay conditions. [c] The activity is reported as the increase in absorbance at 440 nm per hour at pH 5.5 and at 40 °C under the assay conditions. [d,e] One unit of activity corresponds to the liberation of 1 μmol of *p*-nitroaniline/h at pH 7.2[d] or 8.6[e] and at 30 °C under the assay conditions.

Characterization of Digested Proteins by SDS−PAGE. At different times, the reaction was stopped by freezing the suspensions in liquid nitrogen. After freeze-drying, the proteins were examined with SDS−PAGE, as described by Bleukx et al. (1997). Protein samples (2.0 mg) were solubilized in sample buffer (0.25 mL) containing 17.7 mM tris(hydroxymethyl)-aminomethane-HCl (Tris-HCl; pH 6.8), 1% (w/v) SDS, 20% (w/v) sucrose, 5% (v/v) β-mercaptoethanol, and 0.01% (w/v) bromophenol blue. Samples were shaken overnight (7 °C), re-equilibrated to room temperature, and clarified by centrifugation (11000*g*, 3 min).

Samples (30 μL) were submitted to SDS−PAGE (Laemmli, 1970) in a SE 600 Series gel electrophoresis unit (Hoefer Pharmacia Biotech Inc., San Francisco, CA). The slab gels (14.0 × 16.0 × 1.5 cm) consisted of a stacking gel (3.88%T, 1.33%C) and a running gel (17.57%T, 0.455%C). Running conditions were 18 °C and 30 mA. Separation was stopped when the tracking dye ran off the gel. The molecular mass markers were phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa).

Gels were stained overnight with 0.025% Coomassie brilliant blue R250 in 40% methanol containing 7% acetic acid. Destaining was with a 5% methanol solution containing 7% acetic acid until the background was clear.

RESULTS AND DISCUSSION

Characteristics of the Whole Rye Meal Extracts. Sodium acetate buffer (0.05 M, pH 5.0) was the best extraction buffer for solubilization of the proteolytic enzymes in rye whole meal with Humbolt as test material and hemoglobin as substrate (results not shown). Other solvents, such as sodium phosphate buffer and dilute acetic acid, yielded lower proteolytic activities. This is consistent with previous reports for wheat flours that proteolytic enzymes (McDonald and Chen, 1964), especially aspartic proteases (Timmermann and Belitz, 1993), are optimally extracted with sodium acetate buffers at acidic pH values. A concentration of 1:10 (w/v) was chosen to obtain extracts of low viscosity.

Table 1 shows that endoproteolytic, exoproteolytic, carboxypeptidase, aminopeptidase, and BAPA hydrolyzing activities are found in the three rye varieties. The highest proteolytic activities toward hemoglobin, azocasein, and CPA were found in the extract of Humbolt. Aminopeptidase activities were almost the same for the three varieties, whereas the BAPA hydrolyzing activity was significant higher in the Halo extract. Determination of the protein content showed that there was more protein material in the Humbolt extract (1.34 mg/mL) than in those of Amando and Halo (1.20 mg/mL). Because the extraction of Humbolt yielded the highest proteolytic activity, this variety was used for further analysis.

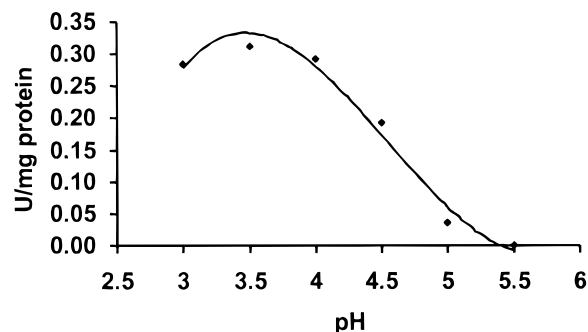

Figure 1. Humbolt (RWME) hemoglobin hydrolyzing activity versus pH profile at 40 °C in 0.2 M sodium acetate buffer (pH range 3.0−5.5). One unit of activity corresponds to the enzyme activity that liberates 1 mg of leucine/h under the assay conditions.

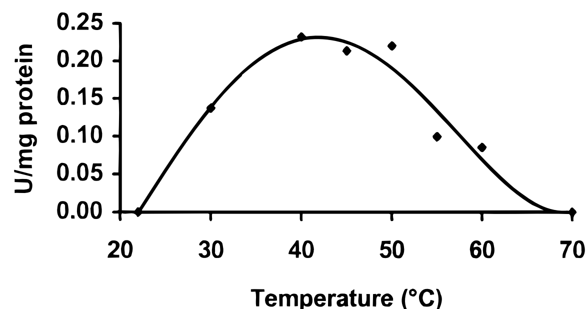

Figure 2. Humbolt (RWME) hemoglobin hydrolyzing activity versus temperature profile in 0.2 M sodium acetate buffer (pH 3.5) in the temperature range 22−70 °C. One unit of activity corresponds to the enzyme activity that liberates 1 mg of leucine/h under the assay conditions.

The pH optimum of the hemoglobin hydrolyzing activity of Humbolt extracts was ∼3.5 (Figure 1). Similar profiles were obtained with the Amando and Halo extracts. The activity steadily declines with an increase in pH. Above pH 5.0, only 10% of the original activity was present. Similar results were reported for wheat (McDonald and Chen, 1964) and triticale proteases (Singh and Katragadda, 1980).

The temperature optimum of Humbolt RWME for hemoglobin hydrolysis was ∼40−45 °C (Figure 2). After 150 min of incubation at 60 °C, the Humbolt RWME still had 40% of the initial activity. Only 5% of the initial activity was measured on 150 min of incubation at 70 °C.

Rye Milling. Data on the laboratory milling of Humbolt are listed in Table 2. The rather low rye flour yield (51%) was probably due to the fact that the pilot scale apparatus in this work was a wheat flour mill. The milling fractions could be defined as “technological” fractions containing mainly one type of tissue but also contaminated by others. However, they could be classified from inside to outside parts of the grain as follows: break flour (B fractions), reduction flour (C fractions), shorts, and bran (Bonnin et al., 1998). The data obtained are in line with those of Drews and Seibel (1976) and Delcour et al. (1989), who found that flours from the inner endosperm (break streams) are relatively rich in starch and poor in protein.

Table 2. Laboratory Milling of Humbolt Rye Sample

fraction	yield[a] (%)	nitrogen content[a] (%)	ash content[a] (%)	hemoglobin hydrolyzing activity[b] (units/g of milled fraction)	azocasein hydrolyzing activity[c] (units/g of milled fraction)
whole kernel	100	2.27	2.05	3.91	1.40
B1	9.2	1.12	0.52	0.20	0.51
B2	11.7	1.60	0.60	0.77	1.08
B3	4.1	2.13	0.98	1.71	1.40
C1	17.1	1.73	0.68	0.55	1.21
C2	6.3	2.09	0.91	2.29	1.48
C3	2.7	1.95	0.90	1.43	1.52
shorts	33.2	2.79	2.74	7.44	2.70
bran	15.8	3.11	5.25	6.49	3.03

[a] All results are reported as percentages of dry matter. [b] One unit of activity corresponds to the liberation of 1 mg of leucine/h at pH 4.0 and 40 °C under the assay conditions. [c] The activity is reported as the increase in absorbance at 440 nm per hour at pH 5.5 and at 40 °C under the assay conditions.

Table 3. Effect of Different Inhibitors on the 35−60% Ammonium Sulfate Fraction from Humbolt Rye Bran Using Hemoglobin and Azocasein as Substrates

inhibitor	protease classes inhibited	concentration	% activity against hemoglobin	% activity against azocasein
none			100	100
ethanol		2%	99	99
EDTA	metallo	5.0 mM	97	93
pepstatin A[a]	aspartic	0.1 mM	12	25
PMSF[a]	serine	2.0 mM	67	67
TLCK	serine (trypsin)	1.0 mM	97	103
TPCK[a]	serine (chymotrypsin)	1.0 mM	90	91
p-HMB	cysteine (some serine)	1.0 mM	95	54
leupeptin	cysteine (some serine)	0.02 mM	94	97
NEMI[a]	cysteine	2.0 mM	95	84
iodoacetamide	cysteine	1.0 mM	94	92
E-64	cysteine	0.05 mM	96	97

[a] Inhibitors dissolved in ethanol such that the concentration in the final assay was 2%.

Hemoglobin and azocasein hydrolyzing activities were much higher in the bran and the shorts than in the different flour fractions. Activities in the bran and shorts extracts were also higher than in Humbolt RWME. Similar results were found for triticale (Madl and Tsen, 1973). The proteolytic activity in the different B-milling fractions increased as the proportion of flour from the outer parts increased for both substrates. For the different C-milling fractions, the same increase was not found with hemoglobin as substrate. Hemoglobin hydrolyzing activity was higher in the C2-milling fraction than in the C3-milling fraction. Assays of the milling fractions indicated that proteolytic activities of the fractions increased steadily as the nitrogen contents of the fractions increased. A significant correlation was also found between the ash content of the different milling fractions and their proteolytic activities (for $n = 9$; $R^2 = 0.78$; $P \leq 0.001$), indicating that the different milling fractions contain different levels of the bran fraction. After all, the bran fraction had the highest proteolytic activity and the highest ash content.

Because the bran fraction had higher proteolytic activity against azocasein, this fraction was chosen for further experiments.

Effect of Inhibitors on Proteolytic Enzymes in Rye. The proteolytic activities in the bran fraction were concentrated using ammonium sulfate. Most of the hemoglobin (80%) and azocasein (90%) hydrolyzing activities were found in the 35−60% AS fraction. This is in agreement with earlier studies indicating that most of the wheat (Timmermann and Belitz, 1993; Bleukx et al., 1998) and barley (Poulle and Jones, 1988) proteolytic enzymes precipitate between 35 and 60% AS saturation.

The effects of different protease inhibitors on the activities of the concentrated AS fraction of rye bran at the given pH levels are summarized in Table 3. Pepstatin A, an inhibitor of aspartic proteases, reduced ~88 and ~75% of the hemoglobin and azocasein hydrolyzing activities, respectively. PMSF, an inhibitor of serine proteases, inhibited ~33% of the same activities. These results are in line with those of Timmermann and Belitz (1993) for resting wheat seeds, where aspartic proteases were the most abundant proteases.

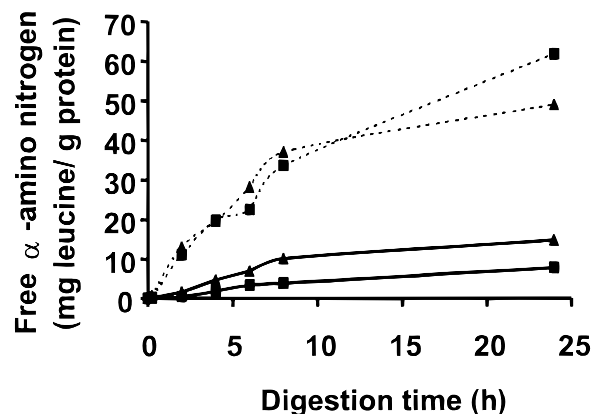

Figure 3. Increase of free α-amino nitrogen of secalins (▲) and gluten proteins (■) as a function of time by adding Humbolt RWME (—) and the 35−60% AS rye bran fraction (- - -) at pH 4.0 and 40 °C.

Tetrasodium ethylenediamine tetraacetate (EDTA) had no significant effect on the activity of the protein hydrolyzing enzymes. The proteolytic activity against azocasein was inhibited by 46% with *p*-hydroxymercuribenzoic acid (*p*-HMB), an inhibitor of cysteine proteases. The fact that *p*-HMB and, to a lesser degree, leupeptin inhibited the proteolytic activity, whereas *N*-ethylmaleimide (NEMI), iodoacetamide, and *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64)

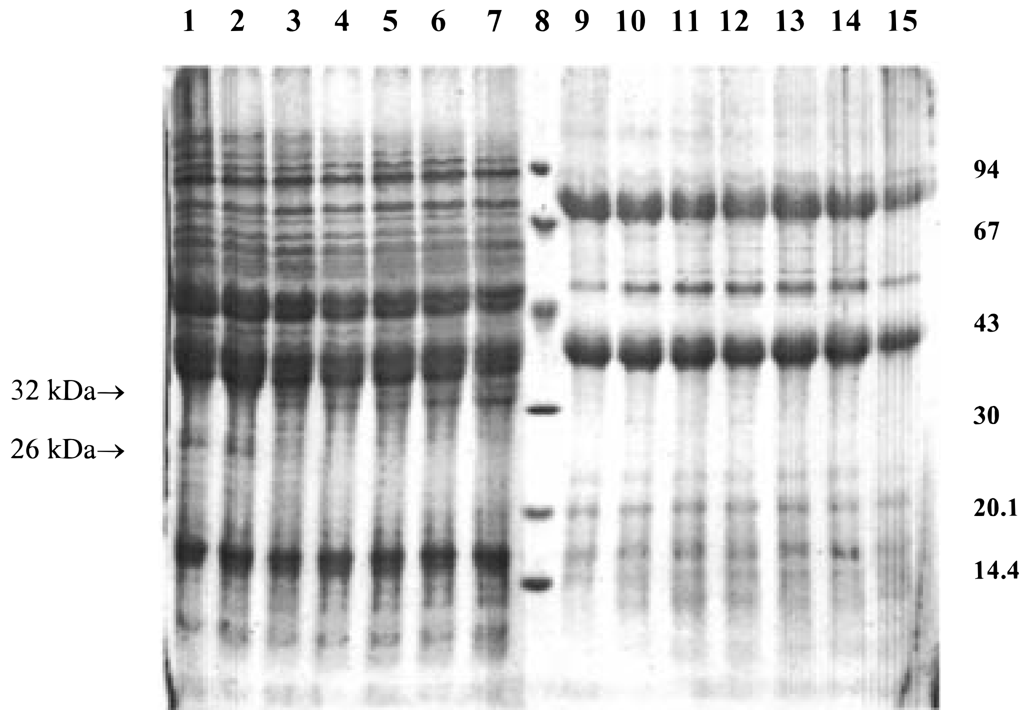

Figure 4. SDS−PAGE patterns of digested commercial gluten proteins (lanes 1−7) and secalins (lanes 9−15) by Humbolt RWME as a function of time. Enzyme solution and storage proteins were incubated for 0.25 h (lanes 2 and 10), 2 h (lanes 3 and 11), 4 h (lanes 4 and 12), 6 h (lanes 5 and 13), 8 h (lanes 6 and 14), and 24 h (lanes 7 and 15). Control samples (lanes 1 and 9) were incubated for 24 h without the addition of the enzyme solution. Lane 8 contains molecular mass markers.

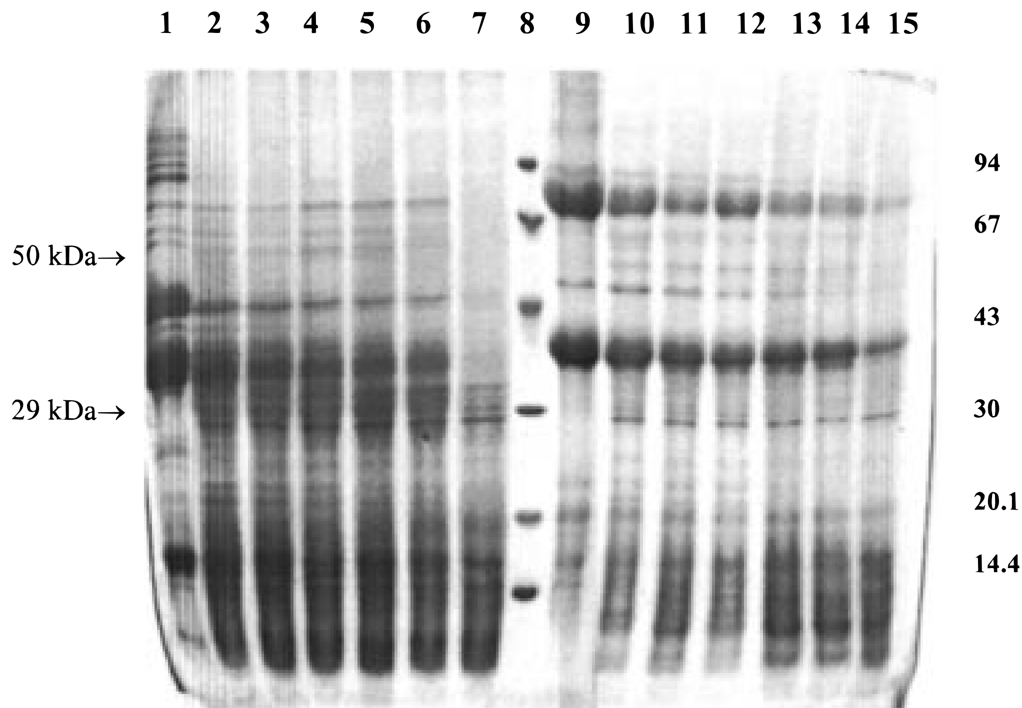

Figure 5. SDS−PAGE patterns of digested commercial gluten proteins (lanes 1−7) and secalins (lanes 9−15) as a function of time by a concentrated 35−60% AS fraction of rye bran. Enzyme solution and storage proteins were incubated for 0.25 h (lanes 2 and 10), 2 h (lanes 3 and 11), 4 h (lanes 4 and 12), 6 h (lanes 5 and 13), 8 h (lanes 6 and 14), and 24 h (lanes 7 and 15). Control samples (lanes 1 and 9) were incubated for 24 h without the addition of the enzyme solution. Lane 8 contains molecular mass markers.

did not, does not necessarily imply that one of the enzymes was a cysteine protease. Examples of serine proteases inhibited by *p*-HMB, but not by other cysteine protease inhibitors, are known in the literature (Umetsu et al., 1981; Bleukx et al., 1997). The hydrolysis of hemoglobin and azocasein may well be the result of the action of aspartic and serine proteases.

Evaluation of the Effect of RWME and the 35−60% AS Rye Bran Fraction on Secalins and Gluten. *Increase of Free α-Amino Nitrogen as a Function of Time.* When adding the enzyme extracts to gluten and secalins, we noticed an increase in the free α-amino nitrogen content as a result of hydrolysis of the substrates (see Figure 3). The increase was most pro-

nounced for the concentrated bran 35–60% AS fraction with both secalins and gluten as substrate. The RMWE had clearly more affinity for the secalins than for gluten, as it released more α-amino nitrogen of the secalins than of the gluten as a function of time.

By making use of inhibitors it was clear that under the experimental conditions, both substrates were hydrolyzed by the same proteolytic enzymes that degraded azocasein and hemoglobin, that is, aspartic and serine proteases.

Characterization of Digested Proteins by SDS–PAGE. SDS–PAGE patterns after different periods of storage protein hydrolysis (Figures 4 and 5) differed for both enzyme extracts. With RWME, small differences were visible on the gel as a function of time. For gluten, we noticed the appearance of a protein band around 32 kDa and the disappearance of the high molecular weight (HMW) glutenin subunits. Also, a protein band around 26 kDa disappeared after incubation at 40 °C. With secalins as substrate, we noticed new protein bands around 40 and 50 kDa.

Comparable and at the same time clearer results were found when the concentrated 35–60% AS fraction of the rye bran was added. For gluten, after 15 min of incubation, all HMW glutenin subunits were degraded and new protein bands were formed with molecular masses around 30 kDa. A clear protein band was formed with a molecular mass of 29 kDa. After 24 h of incubation, almost all proteins with molecular masses >30 kDa were hydrolyzed. Only the protein bands with molecular masses of 29 and 43 kDa remained. With secalins as substrate, the results were again clearer than with Humbolt RWME. We first noticed a protein band with a molecular mass around 50 kDa that disappeared later on. Protein bands of low molecular mass were formed.

CONCLUSIONS

This study demonstrates the presence of proteolytic enzymes in dormant seeds of rye (*S. cereale* L.). Endoproteolytic, exoproteolytic, carboxypeptidase, aminopeptidase, and *N*-α-benzoylarginine-*p*-nitroanilide hydrolyzing activities were found in RWME of three varieties. Further analysis showed that the proteolytic enzymes were especially found in the bran and the shorts fractions.

The proteolytic enzymes of the Humbolt bran fraction were concentrated with ammonium sulfate, and studies with inhibitors revealed that aspartic and serine proteases were responsible for hemoglobin and azocasein hydrolyzing activities at pH 4.0. The same classes of proteases were found in resting seeds of wheat (Dominguez and Cejudo, 1995) and barley (Wrobel and Jones, 1992).

Under our experimental conditions (including urea treatment of secalins and boiling of gluten), both rye and wheat storage proteins were degraded by RWME and a fraction obtained via ammonium sulfate concentration from rye bran. Thus, in practical applications, such as the production of traditionally prepared wheat bread containing 30% rye, it is not unlikely that rye proteases influence the structure of the gluten proteins.

The isolation and purification of new proteolytic enzymes from rye that can degrade gluten proteins is now in progress.

ABBREVIATIONS USED

AS, ammonium sulfate; BAPA, *N*-α-benzoylarginine-*p*-nitroanilide; CPA, *N*-carbobenzoxy-L-phenylalanyl-L-alanine; E-64, *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane; EDTA, tetrasodium ethylenediamine tetraacetate; *p*-HMB, *p*-hydroxymercuribenzoic acid; LPA, L-leucine-*p*-nitroanilide; NEMI, *N*-ethylmaleimide; PMSF, phenylmethanesulfonyl fluoride; RWME, rye whole meal extract; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; TLCK, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone; TNBS, trinitrobenzenesulfonic acid; TPCK, *N*-α-*p*-tosyl-L-phenylalanine chloromethyl ketone.

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