Degradation of Gluten by Proteases from Dry and Germinating Wheat (*Triticum durum*) Seeds: An in Vitro Approach to Storage Protein Mobilization

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Vital gluten was used as an ideal substrate to investigate the role of some proteases in storage protein degradation. Aspartic proteinase and carboxypeptidase were identified as endogenous enzymes adsorbed on gluten and their optimum pH values determined. SDS−PAGE of soluble products released by gluten digestion revealed that the activity of these proteases plays a minor role in protein mobilization, whereas cysteine proteinase, purified from wheat seeds at the fourth day of germination, is extremely effective, producing a remarkable protein degradation in short times. Synergistic effects of aspartic and cysteine proteinase were not observed. Spin labeling of the sulfhydryl groups of gluten proteins enabled a comparative EPR investigation of the consequences of proteolytic degradation on gluten elasticity. It was found that storage protein mobilization brings a loss of elasticity to the polymeric network of gluten, which is particularly marked when the hydrolysis is performed by cysteine proteinase.

Keywords: *Gluten; storage protein mobilization; spin labeling EPR; proteases; Triticum durum; seeds*

INTRODUCTION

Seed storage proteins represent a rich reservoir of nitrogen, sulfur, and carbon for the growth of cereal seedlings prior to the development of autotrophy. In wheat seeds, storage proteins are mainly concentrated (∼80%) in the starchy endosperm (Cornell and Hoveling, 1997). Because these proteins are insoluble in water, their utilization by the growing embryo is possible only after their degradation to soluble products.

Aspartic proteinases, already present in dry seeds, were considered to be responsible for the first step of protein mobilization by some authors (Dunaevsky et al., 1989; Belozersky et al., 1989), whereas others attributed a major role to cysteine proteinases, synthesized de novo during seed germination (Bottari et al., 1996; Galleschi, 1998; Sutoh et al., 1999). The first hypothesis was supported by the results of in vitro attacks of gliadins with aspartic proteinase and carboxypeptidase, two proteolytic enzymes present in the starchy endosperm of dry wheat seeds (Belozersky et al., 1989; Sarbakanova et al., 1987). In fact, it was found that carboxypeptidase had no hydrolytic capacity, whereas aspartic proteinase weakly degraded γ- and ω-gliadins after long incubation times, the degradation increasing by ∼25% when the two enzymes were simultaneously present. Moreover, the addition of aspartic proteinase and carboxypeptidase increased by 3−4 times the rate of digestion of gliadins by cysteine proteinase. On the other hand, the same authors (Belozersky et al., 1989) report that the gliadins were completely hydrolyzed by cysteine proteinase after 15 h of incubation. In agreement with this last finding, in a previous paper by one of the authors (Bigiarini et al., 1995) it was demonstrated that the bulk of proteolytic activity (∼90%) during wheat germination is due to cysteine proteinase. A key role of this class of enzymes in storage protein mobilization was also reported for seeds of other cereals, such as maize (Barros and Larkins, 1990), barley (Koehler and Ho, 1990; Zhang and Jones, 1996), and rice (Kato and Minamikawa, 1996). Nevertheless, a full description of the specific and synergistic action of aspartic proteinase, carboxypeptidase, and cysteine proteinase during wheat seed germination is not available at present, and this limits our knowledge of one of the key events during seedling formation, that is, the mobilization of storage proteins. Moreover, although there are several studies on the proteolytic enzymes of wheat (McDonald and Chen, 1964; Wang and Grant, 1969; Kaminski et al., 1969; Kruger, 1971; Stauffer, 1987; Lin et al., 1993), further research on this topic is needed.

To this purpose, in the present work vital gluten was used as a substrate for in vitro simulations of the action

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10.1021/jf0006170 CCC: $19.00
Published on Web 11/14/2000

of seed proteases. Gluten indeed represents an ideal material for investigating proteolytic degradation because it contains ~85% of the total endosperm proteins (Cornell and Hoveling, 1997), closely resembles the natural target of endogenous proteases, and maintains the polymeric structure of the native storage proteins. In addition, it contains aspartic proteinase and carboxypeptidase activities (Bleukx et al., 1997). Here, the presence of these enzymes adsorbed on vital gluten was verified and their optimum pH values, close to the in vivo pH (~4) of the starchy endosperm during wheat germination (Hamabata et al., 1988), were determined by means of specific enzymatic assays. Thus, two complementary approaches were followed to investigate the hydrolysis of gluten by proteases from dry and germinating wheat seeds, that is, aspartic proteinase, carboxypeptidase, and cysteine proteinase.

In the first one, gel electrophoresis (SDS−PAGE) was applied to characterize the products released by gluten to an incubation buffer during the attack of the different proteases. Suitable inhibitors and incubation procedures were employed to distinguish the action of each enzyme. At the same time, the degradation of gluten by proteases was monitored by spin-labeling EPR (Freed, 1976; Schneider and Freed, 1989), a technique particularly useful for achieving information on the structure and dynamics of gluten proteins (Hargreaves et al., 1994, 1995; Pinzino et al., 1999), two microscopic properties that were found to be related to gluten elasticity (Hargreaves et al., 1994). Here, EPR has to be preferred to more direct rheological methods for following proteolysis because the modest quantities of cysteine proteinase available impose the utilization of small amounts of gluten. For the EPR study, samples of gluten were labeled at its sulfhydryl groups (cysteine residues) with 3-MAL (Keana, 1978) and treated with the various proteases. Line shape analyses of the spectra recorded on these samples between 0 and 90 °C revealed changes in the dynamics of the spin labels after gluten digestion, which were explained in terms of structural modification of the gluten network affecting the cysteine residues environments. [Labeling with 3-MAL does not modify the properties of gluten as proved by the fact that no differences in proteolytic activities adsorbed on gluten and in the susceptibility of gluten to the attack of cysteine proteinase were observed with respect to a control (data not shown).]

MATERIALS AND METHODS

Materials. Flour of *Triticum durum* L. (Gramineae) cv. Lira, obtained from the Società Produttori Sementi (Bologna, Italy), was utilized. Vital gluten was prepared with Glutomatic 2100 (Perten) following the procedure described in the operation manual. Distilled water was used for washing the dough instead of a 2% NaCl solution. The gluten so obtained was frozen, lyophilized, homogenized in dry conditions with a pestle and mortar, and stored at −20 °C. The vital gluten had a moisture content of ~6%.

Cysteine proteinase was purified from germinating wheat seeds following the procedure reported by Bottari et al. (1996).

Pepstatin A, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64), hemoglobin (Hb), and 3-maleimidoproxyl (3-MAL) were purchased from Sigma (St. Louis, MO). Pepstatin A and E-64 were utilized in final concentrations of 1.46 and 20 μM, respectively.

All reagents were of analytical grade and used without further purification.

Spin Labeling of Gluten. Spin labeling of gluten was performed following the procedure reported by Pinzino et al. (1999), but without defatting the flour. The excess of 3-MAL was removed by washing with distilled water until no detectable amounts of the spin label were revealed in the washing water by HPLC analysis (Jasco 880 Pu pump equipped with a Jasco 875-UV spectrophotometric detector; RP-C18 column; 6 min and 40 s of retention time of 3-MAL at 0.6 mL/min flow rate; mobile phase $CH_3CN/H_2O/CF_3COOH$ 63:37:0.05; $\lambda = 230$ nm). Labeled gluten was freeze-dried and stored at −20 °C.

Gluten Autodigestion and Hydrolysis by Cysteine Proteinase. Gluten was reconstituted with distilled water at room temperature to form a cohesive elastic ball.

For autodigestion, 100 mg of reconstituted gluten was suspended in 1 mL of 0.2 M sodium acetate buffer (pH 4) containing 2.5 mM 2-mercaptoethanol. 2-Mercaptoethanol, although unnecessary in gluten autodigestion, was added to allow a direct comparison with the hydrolysis of gluten by cysteine proteinase, in which it guarantees the enzyme activity. Nevertheless, the mild concentration and temperature conditions used prevent any relevant reduction of disulfide linkages of gluten proteins. The autodigestion was performed both in the absence and in the presence of pepstatin A (1.46 μM final concentration). The preparations were incubated at 30 °C under continuous shaking. At different times during the incubation, the gluten residue was removed by centrifugation at 40000*g* and 4 °C for 10 min, and the supernatants were assayed for proteolytic activity and utilized for SDS−PAGE analyses.

Gluten hydrolysis by cysteine proteinase was performed following the same procedure adopted for autodigestion, except that 150 μL (2.65 units) of enzyme solution was added to reconstituted gluten in the place of the same amount of buffer. Pepstatin A and E-64 (1.46 and 20 μM final concentrations, respectively) were used as inhibitors. The preparations were incubated at 30 °C under continuous shaking. At different times during the incubation, the gluten residue was removed by centrifugation at 40000*g* and 4 °C for 10 min, and the supernatants were assayed for proteolytic activity and utilized for SDS−PAGE analyses. In some experiments gluten was preliminarily autodigested for 6 h either in the absence or in the presence of pepstatin A and then centrifuged (10 min at 40000*g* and 4 °C), and the supernatants (0.8 mL) were incubated for 120 min with cysteine proteinase in the above-described conditions. The incubations were performed either in the absence or in the presence of pepstatin A and E-64 (1.46 and 20 μM final concentrations, respectively).

Labeled Gluten Autodigestion and Hydrolysis by Cysteine Proteinase. For EPR analyses of the released proteins during gluten autodigestion, gluten was reconstituted with distilled water at room temperature to form a cohesive elastic ball. Thus, 100 mg of reconstituted labeled gluten was suspended in 1 mL of 0.2 M sodium acetate buffer (pH 4) containing 2.5 mM 2-mercaptoethanol. The preparation was incubated at 30 °C under continuous shaking. At different times during the incubation, the gluten residue was removed by centrifugation at 40000*g* and 4 °C for 10 min, and the supernatants were collected for EPR analyses.

For EPR measurements on autodigested gluten, 50 mg of labeled gluten was reconstituted with 38 μL of 1 M sodium acetate buffer (pH 4) containing 5 mM 2-mercaptoethanol and 63 μL of distilled water. The obtained cohesive elastic ball was put into a plastic microtest tube and incubated for 6 h at 30 °C. The digestion was stopped at different times during the incubation by immersion of the microtest tube in liquid nitrogen, and samples for EPR measurements were collected. The autodigestion of labeled gluten was also performed in the presence of pepstatin A (1.46 μM final concentration).

The hydrolysis of labeled gluten by cysteine proteinase was performed following the same procedure, except that 63 μL (1.1 units) of the enzyme solution was added to gluten instead of distilled water. The hydrolysis of labeled gluten by cysteine proteinase was performed either in the absence or in the presence of E-64 (20 μM final concentration).

SDS—PAGE. For electrophoretic analyses protein solutions (100 μL) deriving from gluten autodigestion or enzymatic hydrolysis by cysteine proteinase were collected and freeze-

dried. Analyses were performed by discontinuous SDS−PAGE according to the method of Laemmli (1970), using a 12% resolving gel and a 4% stacking gel. Freeze-dried proteins were dissolved in 1 mL of 62 mM Tris-HCl buffer (pH 6.8) containing 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, and 0.1% Bromophenol Blue, boiled for 3 min, and loaded (5 μL) on SDS gels. Molecular weight standards used included β-galactosidase (116.3 kDa), phosphorylase *b* (97.4 kDa), ovotransferrin (77.0 kDa), bovine serum albumin (66.25 kDa), glutamate dehydrogenase (55.0 kDa), ovalbumin (42.7 kDa), carbonic anhydrase (30.0 kDa), myoglobin (17.2 kDa), and cytochrome *c* (12.3 kDa). The gels were stained overnight with Coomassie Brilliant Blue R250 (Koenig et al., 1970), destained with 7.5% (v/v) acetic acid, scanned, and processed by a digital image analysis program (SigmaGel, Jandel Corp.).

Determination of Optimum pH Values for Aspartic Proteinase and Carboxypeptidase Activities. The aspartic proteinase activity was measured by using 1% (w/v) Hb dissolved in McIlvaine buffer at various pH values ranging from 1.5 to 6.5. One hundred and fifty microliters of supernatant solution was mixed with 850 μL of McIlvaine buffer containing 2-mercaptoethanol (final concentration = 2.5 mM). The reaction was started by adding 1 mL of hemoglobin, incubated at 30 °C for 12 h, and stopped by adding 1 mL of 24% (w/v) trichloroacetic acid (TCA) and vortexing. After standing at 4 °C for 10 min, the obtained suspension was cleared by centrifugation, and 0.5 mL of the supernatant was utilized to determine the TCA-soluble products according to the ninhydrin method (Yemm and Cocking, 1955). Controls were treated in the same manner but without incubation.

The carboxypeptidase activity was measured with 5 mM *N*-carbobenzoxy-L-phenylalanyl-L-alanine (CBZ-Phe-Ala) dissolved in McIlvaine buffer at various pH values ranging from 1.5 to 6.5. Fifty microliters of supernatant solution was mixed with 950 μL of McIlvaine buffer containing 2-mercaptoethanol (final concentration = 2.5 mM), and the reaction was started by adding 1 mL of CBZ-Phe-Ala. After 90 min of incubation, the reaction was stopped by adding 1 mL of 24% (w/v) TCA and vortexing. The suspension was centrifuged, and 0.5 mL of the supernatant was utilized for the determination of released alanine according to the ninhydrin method (Yemm and Cocking, 1955).

Protein Estimation. The concentration of proteins was evaluated following the Lowry method as modified by Bensadoun and Weinstein (1976). Bovine serum albumin was used as a standard, and the measurements were performed in a Perkin-Elmer 550S UV−vis spectrophotometer.

EPR Measurements. EPR spectra were recorded using a Varian E112 spectrometer (X-band) equipped with a Varian E257 temperature control unit. The spectrometer was interfaced to a 100-MHz personal computer by means of a home-made data acquisition system, consisting of an acquisition board (Ambrosetti and Ricci, 1991) and a software package especially designed for EPR and ENDOR experiments (Pinzino and Forte, 1992). For each spectrum a field setting of 3265 G, a microwave power setting of 5 mW, and modulation amplitude of 1 G were employed.

Spectra of gluten samples submitted to autodigestion or hydrolysis by cysteine proteinase were recorded every 10 °C between 0 and 90 °C on temperature rising, with a 15 min delay for temperature equilibration. For each sample, ~15 mg of treated gluten was taken up into a quartz capillary (1 mm internal diameter), which was inserted into a quartz sample holder (4 mm external diameter) closed to both extremities to avoid sample loss.

Spectra on proteins released by gluten to the buffer during autodigestion were recorded at room temperature. Samples were enclosed in quartz tubes with 2 mm internal diameter.

RESULTS AND DISCUSSION

Gluten Autodigestion. When vital gluten was incubated in an acetate buffer (pH 4) containing 2.5 mM 2-mercaptoethanol, a gradual release of soluble proteins in the medium was observed. In Figure 1 the proteins released by the gluten are reported as a function of incubation time; an increasing trend is shown up to 120 min. The same results were obtained when the incubation of gluten was performed in the absence of the reducing agent (data not shown). Longer incubation times (up to 720 min) were employed in autodigestion assays of spin-labeled gluten. In this case, the release of proteins in the medium was followed by measuring the signal amplitude in the EPR spectra of supernatants isolated from the incubation mixtures. As shown in Figure 2, the amplitude (*A*), normalized for the weight of the supernatants, shows as a function of the autodigestion time (*t*) a trend typical of a pseudo-first-order reaction, which can be expressed in terms of the equation

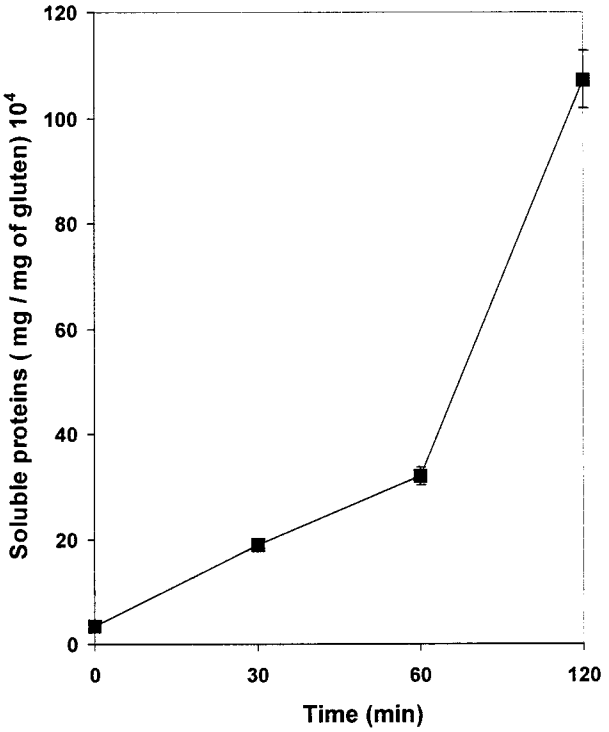

Figure 1. Released proteins at different incubation times during gluten autodigestion (pH 4, 30 °C), evaluated as milligrams of proteins per milligram of gluten. Each value is an average of three replicates of at least two extractions.

$$A = A_\infty[1 - \exp(-k(t + t_m))] \quad (1)$$

where t_m is a dead time accounting for the experimental procedure and k is the kinetic constant for the release of proteins in the incubation medium. The optimized value of k found by applying a fitting procedure to the experimental data is 0.28 h^{-1} (correlation coefficient = 0.9973, t_m = 4.88 min).

Among released proteins, two proteolytic enzymes were detected by utilizing exogenous substrates, that is, Hb and CBZ-Phe-Ala (see Figure 3). The enzyme degrading hemoglobin was inhibited by pepstatin A, whereas that hydrolyzing CBZ-Phe-Ala was inhibited by phenylmethanesulfonyl fluoride (PMSF). These properties allowed the assignment of the two proteolytic enzymes as the aspartic proteinase and carboxypeptidase, previously isolated from wheat seeds (Belozersky et al., 1989; Sarbakanova et al., 1987). Furthermore, using Hb and CBZ-Phe-Ala as substrates, the optimum pH values for the aspartic proteinase and carboxypeptidase activi-

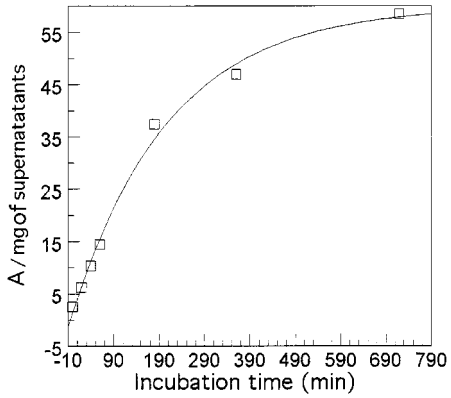

Figure 2. Signal amplitude (*A*) in the EPR spectrum of supernatants collected at different incubation times during the autodigestion (pH 4, 30 °C) of gluten spin labeled with 3-MAL: (squares) measured signal amplitudes normalized for the supernatants weight; (solid line) amplitudes calculated in terms of a pseudo-first-order kinetic law as described in the text.

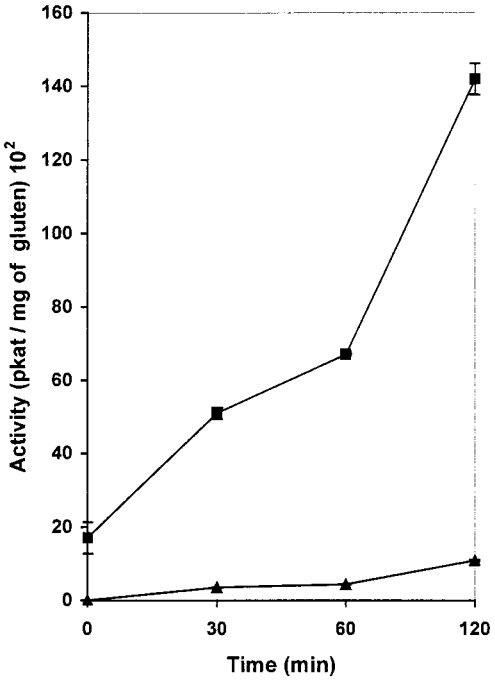

Figure 3. Proteinase and carboxypeptidase activities released during gluten autodigestion (pH 4, 30 °C) assayed with Hb (triangles) and CBZ-Phe-Ala (squares), respectively. Each value is an average of three replicates of at least two extractions.

ties were determined (see Figure 4). The Hbase activity showed two peaks with maxima at pH 3.2 and 5.0, respectively. The intensity of the first peak slightly decreased in the presence of PMSF, indicating that at pH ≅3 the carboxypeptidase has a weak hydrolytic capacity on Hb, whereas aspartic proteinase shows its maximum activity at this pH. On the other hand, the peak with maximum intensity at pH ≅5 can be attributed to the synergistic activity of aspartic proteinase and carboxypeptidase. In fact, it dramatically decreases in the presence of PMSF, indicating that at this pH aspartic proteinase scarcely degrades the hemoglobin, giving hydrolysis products that are further degraded by carboxypeptidase. The assay performed using CBZ-Phe-Ala as a substrate definitively states that carboxypeptidase has its maximum activity at pH 5. Nevertheless,

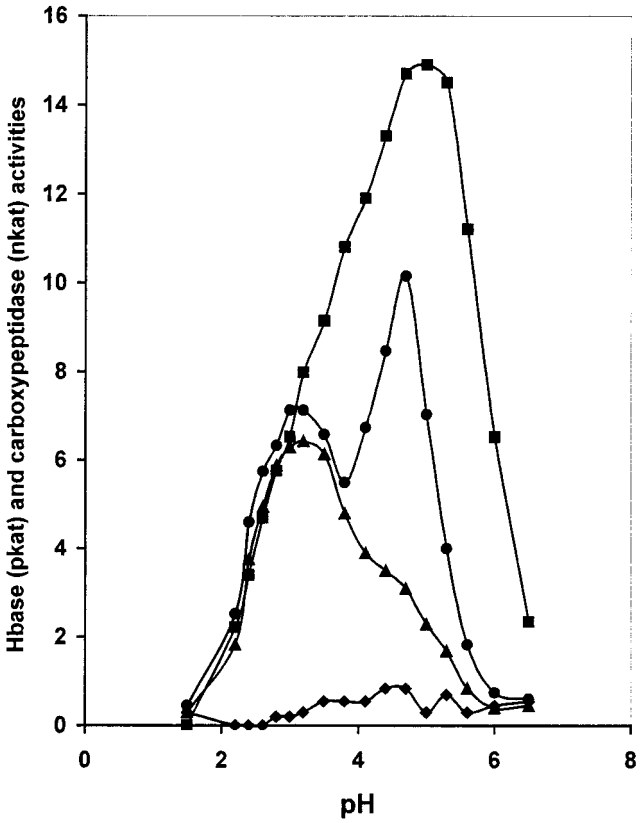

Figure 4. Optimum pH values for proteinase and carboxypeptidase released during gluten autodigestion: (circles) control; (triangles) PMSF; (rhombs) pepstatin A; (squares) CBZ-Phe-Ala. The enzyme activities were assayed at different pH values in a McIlvaine buffer using Hb and CBZ-Phe-Ala as substrates. Values are means of at least three replicates at each pH.

it was observed that the hydrolysis of Hb was almost annihilated when aspartic proteinase was inhibited by pepstatin A, confirming the scarce capability of carboxypeptidase to degrade this protein, and, more generally, native proteins (Sarbakanova et al., 1989), even at its optimum pH.

To further investigate the activity of endogenous enzymes in vital gluten autodigestion, electrophoretic analyses (SDS−PAGE) were performed on the proteins released in the medium at different incubation times, ranging from 1 to 12 h. As can be seen in Figure 5, numerous bands, corresponding to molecular masses ranging from 12 to 106 kDa, characteristic of gliadins and glutenins (Cornell and Hoveling, 1997), were observed in all electrophoretic patterns, with different intensities depending on the incubation time. In fact, the intensity of bands due to some high molecular weight proteins (106, 99, and 92 kDa) decreased with increasing digestion time (see Figure 5a), whereas bands relative to proteins with masses of 76, 66, 62, and 56 kDa showed up and others, associated with proteins with masses of 26, 25, and 24 kDa, disappeared. These small changes in the molecular mass distribution of released proteins were not observed when the autodigestion was repeated in the presence of pepstatin A (see Figure 5b). These findings indicate that aspartic proteinase and carboxypeptidase adsorbed on vital gluten, and when released in the medium during autodigestion, perform a weak hydrolysis of the gluten, even for long incubation times.

This is also reflected by the small modifications

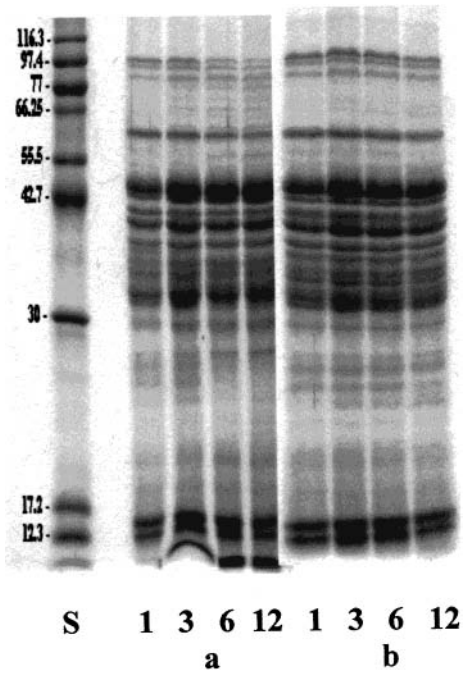

Figure 5. SDS−PAGE of proteins released during gluten autodigestion (pH 4, 30 °C) for 1, 3, 6, and 12 h in the absence (a) and in the presence (b) of pepstatin A. S represents molecular weight standards.

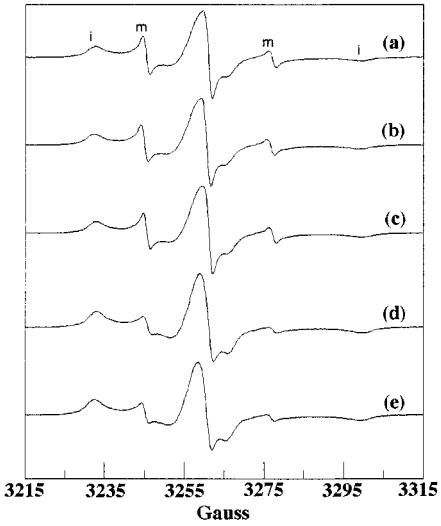

Figure 6. EPR spectra of gluten spin labeled with 3-MAL recorded at 10 °C: (a) untreated gluten; (b) gluten incubated at pH 4 and 30 °C for 6 h; (c) gluten incubated at pH 4 and 30 °C for 6 h in the presence of pepstatin A; (d) gluten incubated at pH 4 and 30 °C for 6 h in the presence of cysteine proteinase; (e) gluten incubated at pH 4 and 30 °C for 6 h in the presence of cysteine proteinase and pepstatin A. "i" and "m" indicate slow- and fast-moving spin label lines, respectively.

induced by these enzymes on the gluten network during autodigestion revealed by means of spin-labeling EPR spectroscopy. In Figure 6 the spectrum recorded at 10 °C on labeled gluten hydrated with an acetate buffer (pH 4) containing 2.5 mM 2-mercaptoethanol to form a cohesive elastic ball (spectrum a) is compared with the spectrum recorded at the same temperature on labeled gluten hydrated in the same manner and incubated for 6 h in order to perform autodigestion without releasing proteins from gluten (spectrum b). Both spectra are of a composite type with line shapes resulting from the superposition of at least two subspectra, each belonging

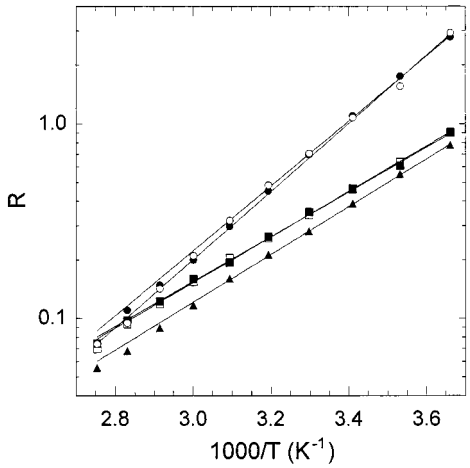

Figure 7. *R* ratios versus 1000/*T*: (triangles) untreated gluten; (solid squares) gluten incubated at pH 4 and 30 °C for 6 h; (open squares) gluten incubated at pH 4 and 30 °C for 6 h in the presence of pestatin A; (solid circles) gluten incubated at pH 4 and 30 °C for 6 h in the presence of cysteine proteinase; (open circles) gluten incubated at pH 4 and 30 °C for 6 h in the presence of cysteine proteinase and pepstatin A. Lines represent fits of experimental *R* values in terms of eq 5.

to spin labels differing in their mobilities. Slow-moving spin labels (immobile) give broad lines in the spectra, whereas sharp lines are attributed to fast-moving spin labels (mobile), the mobility of spin labels being determined by the flexibility of the protein segments to which they are bound, solvent viscosity, and steric hindrance of their environments. The mobile component can be thus attributed to spin labels bound to sulfhydryl groups which allow the labels to retain a high degree of rotational freedom, that is, shallow, solvent-exposed protein groups. On the other hand, the slow-moving component is due to labels bound to sulfhydryl groups localized in somewhat restrictive, crevice-like regions of the proteins. Very subtle differences were observed between the EPR spectra recorded at 10 °C on labeled gluten before and after autodigestion (compare spectra a and b in Figure 6), respectively. At different temperatures similar spectra, but with different intensities of the two subspectra, were observed. Two parameters were calculated from the spectra to obtain quantitative information on spin label dynamics and, in turn, on gluten mobility. The first one is the rotational correlation time (τ) of the mobile spin labels, determined according to the Freed and Fraenkel (1963) equation

$$t = (6.65 \times 10^{10})\Delta h_{+1}[(I_{+1}/I_{-1})^{1/2} - 1] \qquad (2)$$

where Δh_{+1} is the peak-to-peak width of the low-field line, in gauss, and I_{+1} and I_{-1} are the amplitudes of the low- and high-field lines, respectively. The second parameter that can be calculated from the EPR spectra is *R* (Hargreaves et al., 1994, 1995), defined as the ratio

$$R = i/m \qquad (3)$$

where *i* and *m* are the amplitudes of the low-field peaks of the slow-moving and fast-moving spin labels, respectively (see Figure 6). *R* reflects the ratio between slow- and fast-moving spin label populations. Both *R* and τ decreased with increasing temperature (see Figures 7 and 8), indicating that a progressive transfer of less mobile radicals to a more mobile population occurs and that the rotational diffusion of mobile spin labels

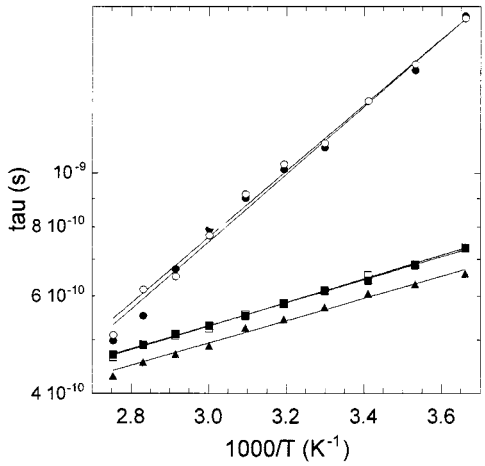

Figure 8. Rotational correlation times, τ, of fast-moving spin labels versus 1000/T: (triangles) untreated gluten; (solid squares) gluten incubated at pH 4 and 30 °C for 6 h; (open squares) gluten incubated at pH 4 and 30 °C for 6 h in the presence of pestatin A; (solid circles) gluten incubated at pH 4 and 30 °C for 6 h in the presence of cysteine proteinase; (open circles) gluten incubated at pH 4 and 30 °C for 6 h in the presence of cysteine proteinase and pepstatin A. Lines represent fits of experimental τ values in terms of eq 4.

becomes faster. Slightly higher values of both R and τ were found at each temperature for autodigested gluten with respect to the untreated one, indicating that autodigestion induces a small increase of rigidity of the polymeric gluten network. The trend of τ as a function of temperature was described in terms of the Arrhenius equation

$$\tau = \tau_\infty \exp(E_a/R_g T) \quad (4)$$

and activation energies for the rotational diffusion of fast-moving spin labels in gluten before and after digestion were determined. Their values are reported in Table 1.

On the other hand, the description of R in terms of the equation

$$R = R_0 \exp(\Delta S/R_g) \exp(-\Delta H/R_g T) \quad (5)$$

where R_0 is a proportionality factor between the label concentration ratio and the spectral amplitude ratio, allowed the determination of the differences in enthalpy (ΔH) and entropy (ΔS) for the transfer of population from mobile to immobile spin labels (see Table 1). In eqs 4 and 5 the temperature is expressed in degrees Kelvin and R_g, the gas constant, is expressed in J/K· mol.

When the autodigestion of spin-labeled gluten was carried out in the presence of pepstatin A, an inhibitor of aspartic proteinases, EPR spectra essentially equal to those of gluten incubated without this inhibitor (compare spectra b and c in Figure 6) were observed. This similarity was reflected by the values of τ and R shown in Figures 7 and 8; an analysis of the trends of these parameters in terms of eqs 4 and 5 gave the activation energy, enthalpy, and entropy values reported in Table 1. These values are substantially identical to those obtained for autodigested gluten. On the basis of these results aspartic proteinase and carboxypeptidase cannot be considered to be responsible for gluten degradation.

Gluten Degradation by Cysteine Proteinase. The results obtained in the experiments of vital gluten autodigestion suggested that other enzymes must be involved in the completion of gluten degradation. On the basis of the results previously reported in the literature on the key role of cysteine proteinase in storage protein mobilization, we decided to investigate the in vitro activity of this enzyme in gluten degradation. Thus, cysteine proteinase was added to the incubation buffer, and gluten hydrolysis was followed by SDS−PAGE on samples of soluble proteins collected at different incubation times, ranging from 30 to 120 min. As shown in Figure 9, the addition of cysteine proteinase caused strong modifications in the molecular mass distribution of the released gluten proteins, even for short incubation times. In particular, the bands corresponding to high molecular weight glutenins (molecular masses from 92 to 106 kDa) and those relative to gliadins (molecular masses from 31 to 47 kDa) almost disappeared in the electrophoretic patterns. At the same time, bands corresponding to proteins with molecular masses of 58 and 38 kDa showed up. Their intensity progressively increased during the incubation, so that they were attributed to gluten hydrolysis products. If the activity of cysteine proteinase was inhibited by E-64, the electrophoretic pattern appeared almost identical to that shown by the control (compare lanes 1 and 3 of Figure 9). On the contrary, the addition of pepstatin A, an aspartic proteinase inhibitor, appeared to be ineffective in blocking gluten degradation (compare lanes 2 and 4 of Figure 9). Similar conclusions could be drawn for all incubation times investigated. These findings indicate that gluten digestion is essentially due to cysteine proteinase.

Further support of these conclusions comes from EPR investigations. In fact, remarkable changes were observed in the spectral line shapes of labeled gluten reconstituted with an acetate buffer (pH 4) containing 2.5 mM 2-mercaptoethanol and cysteine proteinase and incubated for 6 h in comparison with those of both untreated gluten (see spectra a and d in Figure 6) and gluten subjected to autodigestion for 6 h (see spectra b and d in Figure 6). In particular, a bigger population of slow-moving spin labels was shown by the gluten degraded by cysteine proteinase, especially at temperatures <20 °C, indicating that the transfer of slow-moving to fast-moving population is more hindered. From an analysis of the R values in terms of eq 5, ΔH and ΔS values equal to −140.90 J/K mol and −31.97 kJ/mol were found, respectively (see Table 1). Both ΔH and ΔS are greater in absolute value than those previously determined for untreated and autodigested gluten, indicating that in gluten degraded by cysteine proteinase the transferring of spin labels from immobile to mobile populations is more favored by entropic factors and less by enthalpic factors with respect to the case of untreated or autodigested gluten.

The degradation of gluten by cysteine proteinase induces also an increase of τ values, higher at lower temperatures (E_a = 11.64 kJ/mol, as determined by applying the Arrhenius equation). These findings indicate a slowing of the rotational diffusion motion of the mobile spin labels due to a deterioration of the polymeric network of gluten caused by the attack of cysteine proteinase.

Now, the question arises if aspartic proteinase and carboxypeptidase could play a role in protein degrada-

Table 1. Activation Energy (E_a) and Differences of Enthalpy (ΔH) and Entropy (ΔS)[a]

sample	E_a (kJ/mol)	ΔH (kJ/mol)	ΔS (J/ K mol)
untreated gluten	3.85 ± 0.14	−23.52 ± 0.25	−120.64 ± 0.88
gluten after autodigestion for 6 h	3.98 ± 0.05	−22.03 ± 0.35	−114.15 ± 1.25
gluten after autodigestion for 6 h in the presence of pepstatin A	4.08 ± 0.06	−22.43 ± 0.16	−115.44 ± 0.56
gluten after digestion for 6 h with cysteine proteinase	11.66 ± 0.34	−31.93 ± 0.39	−140.83 ± 1.40
gluten after digestion for 6 h with cysteine proteinase in the presence of pepstatin A	11.41 ± 0.26	−33.53 ± 1.10	−146.50 ± 3.96

[a] E_a values were obtained by applying eq 4 to τ values as a function of temperature; ΔH and ΔS values were determined by analyzing the trends of R values as a function of temperature in terms of eq 5.

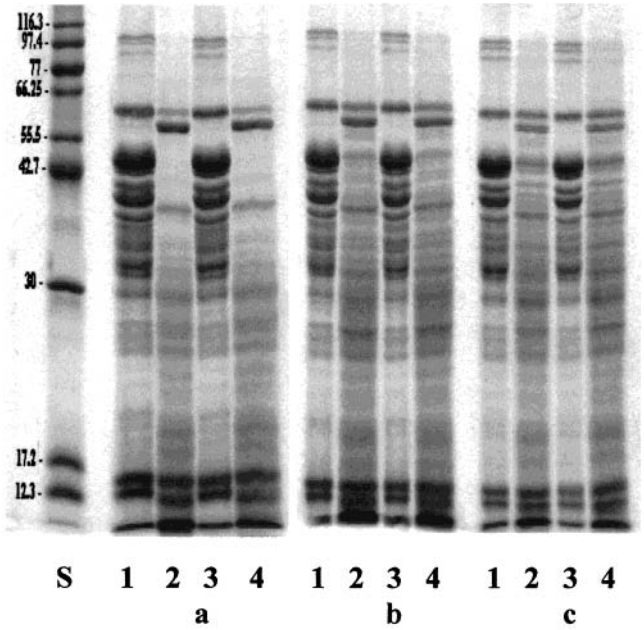

Figure 9. SDS−PAGE of proteins released by gluten after autodigestion or digestion with cysteine proteinase (pH 4, 30 °C) for (a) 30, (b) 60, and (c) 120 min: (lane S) molecular weight standards; (lanes 1) autodigestion; (lanes 2) digestion with cysteine proteinase; (lanes 3) digestion with cysteine proteinase in the presence of E-64; (lanes 4) digestion with cysteine proteinase in the presence of pepstatin A.

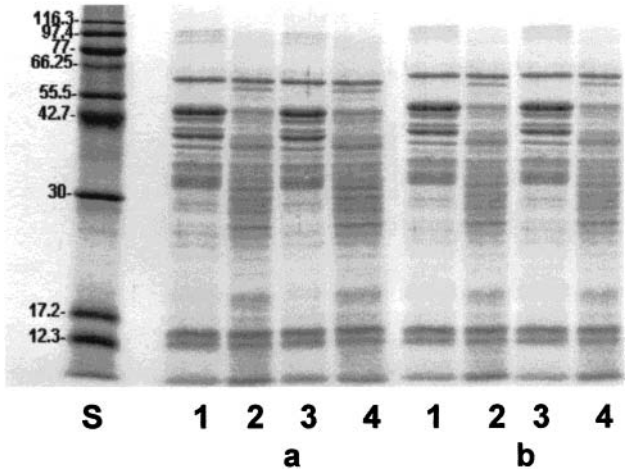

Figure 10. SDS−PAGE of proteins released by gluten in a preliminary autodigestion (pH 4, 30 °C, 6 h) in the absence (a) and in the presence (b) of pepstatin A and subsequent hydrolysis by incubation of the supernatants for 120 min with the cysteine proteinase (2.65 units): (lane 1) released proteins incubated without cysteine proteinase; (lane 2) released proteins incubated with cysteine proteinase; (lane 3) released proteins incubated with cysteine proteinase in the presence of E-64; (lane 4) released proteins incubated with cysteine proteinase in the presence of pepstatin A.

tion prior to the cysteine proteinase action. To reproduce the in vivo situation, in which aspartic proteinase and carboxypeptidase are present in the seed before cysteine proteinase is synthesized during germination, experiments were performed in which gluten was preliminarily autodigested for 6 h and centrifuged, and cysteine proteinase was added to the supernatants and incubated for 2 h. The gluten protein degradation was monitored by SDS−PAGE (see Figure 10a), and the results obtained were very similar to those previously shown for the direct digestion of gluten by cysteine proteinase (see Figure 9). To verify if the action of aspartic proteinase adsorbed to gluten could influence its further hydrolysis by cysteine proteinase, experiments were also performed in the presence of pepstatin A. SDS−PAGE analyses (compare parts a and b of Figure 10) indicate that the presence of pepstatin A during gluten preincubation or during released protein hydrolysis does not affect the degradation performed by cysteine proteinase. Moreover, very similar EPR spectra were obtained for labeled gluten treated with cysteine proteinase whether the autodigestion was inhibited by adding pepstatin A or not (compare spectra d and e in Figure 6). As a consequence, from the spectra recorded on the two samples at the same temperatures, almost equal R and τ values (see Figures 7 and 8) were calculated, from which, in turn, very similar energy, enthalpy, and entropy values were determined applying eqs 4 and 5 (see Table 1). On the basis of these results we can affirm that cysteine proteinase plays a key role in the mobilization of gluten, being able to hydrolyze it without needing a preliminary degradation.

CONCLUSIONS

Vital gluten was employed as a substrate for testing the ability of dry and germinating seed proteases to degrade wheat storage proteins. We believe that gluten represents the best in vitro model substrate for storage protein mobilization in germinating wheat seeds, especially if compared with the groups of single storage proteins (i.e., gliadins or glutenins; Dunaevsky et al., 1989; Bottari et al., 1996; Sutoh et al., 1999) or the exogenous substrates (Dominguez and Cejudo, 1995; Jivotovskaya et al., 1997) used in previous studies reported in the literature.

The consequences of proteolytic attacks on gluten were investigated from two complementary points of view: on the one hand, the molecular mass distributions of proteins released in the incubation medium were monitored by means of electrophoretic analyses; on the other hand, the modifications of the gluten network were studied by spin-labeling EPR.

A massive release of proteins with molecular masses of 38 and 58 kDa was observed from gluten incubated with cysteine proteinase, whereas very small changes in the molecular mass distribution of hydrolyzed proteins were found after gluten autodigestion by aspartic proteinase and carboxypeptidase.

Moreover, gluten degraded by the attack of cysteine proteinase showed an increased rigidity, whereas almost no influence of aspartic proteinase and carboxypeptidase activities was observed on the polymeric structure of gluten proteins.

Insofar as gluten can be assimilated to proteins present in a wheat seed, the results of this study confirm the suggested (Dunaevsky et al., 1989; Shutov and Vaintraub, 1987; Bottari et al., 1996; Jivotovskaya et al., 1997; Sutoh et al., 1999) key role of wheat cysteine proteinases in the degradation of storage proteins. The enzyme would be maintained active in vivo by thioredoxin, which has been demonstrated to increase during the germination of durum wheat (Kobrehel et al., 1992). No hints were found in agreement with the hypothesized action of dry seed proteases at the early stages of seed germination (Dunaevsky et al., 1989). As a consequence, the claimed ability of aspartic proteinase and carboxypeptidase to initiate the degradation in vivo might be a result of the noncompliance of the requirement that storage proteins of ungerminated seeds used in in vitro experiments must be native, because even small structural changes caused by heat or chemical agents may greatly increase their susceptibility to proteolysis (Shutov and Vaintraub, 1987). In fact, the degradation of gliadin was performed in the presence of urea by Dunaevsky et al. (1989).

ABBREVIATIONS USED

CBZ-Phe-Ala, *N*-carbobenzoxy-L-phenylalanyl-L-alanine; E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; EPR, electron paramagnetic resonance; Hb, hemoglobin; 3-MAL, 3-maleimidoproxyl; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride; TCA, trichloroacetic acid.

SAFETY

Precautions were taken to prevent any contact of 3-MAL and labeled gluten with skin and eyes.

ACKNOWLEDGMENT

We thank the Società Produttori Sementi (Bologna, Italy) for supplying the flour.

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Received for review May 18, 2000. Revised manuscript received September 25, 2000. Accepted September 25, 2000. This research was supported by MIPAF, MURST, and CNR (Rome, Italy).

JF0006170