

Effect of *Aelia* spp. and *Eurygaster* spp. Damage on Wheat Proteins

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ABSTRACT

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The effect of *Aelia* spp. and *Eurygaster* spp. wheat bugs on the protein fractions of different wheat cultivars has been studied by size-exclusion high-performance liquid chromatography (SE-HPLC) and free-zone capillary electrophoresis (FZCE). Those methods were used to quantify and characterize the extent of protein modification. A decrease in the amount of alcohol-insoluble polymeric proteins along with an increase in the alcohol-soluble polymeric proteins and gliadins were observed in damaged wheat. The high molecular weight (HMW) and low molecular

weight (LMW) glutenin fractions were barely detected in the incubated damaged wheat from some cultivars, which indicated hydrolysis of those proteins by the bug proteinases. In damaged wheats, both incubated and unincubated, gliadin electrophoregrams revealed the presence of some new peaks with mobilities similar to the ω gliadins. The overall results suggest that the bug proteinases are potent enzymes that appear to be nonspecific because they hydrolyze all gluten proteins.

Wheat is damaged by sucking insects, commonly called bugs, and that damage has produced important economic losses to millers and bakers (Lorenz and Meredith 1988). Bug-damaged wheat has been characterized by a disrupted protein structure due to the action of some injected proteinases (Kretovich 1944), although no alteration in the amylase activities and structure of the starch granules has been observed (Every et al 1990; Rosell et al 2002). Bug-damaged wheat causes reduced flour quality (Karababa and Ozan 1998; and references cited herein), giving a softer dough and subsequently flat bread with low volume and unsatisfactory texture (Lorenz and Meredith 1988; Hariri et al 2000).

In Europe, the Middle East, and North Africa, wheat damage has been attributed to two genera of heteropterous, *Aelia* spp. and *Eurygaster* spp., and in New Zealand, *Nysius huttoni* is considered responsible for the wheat damage (Cressey et al 1987). Spain is one country where bug damage of wheat is considered an endemic problem. Infiesta et al (1999) analyzed the bug damage in the 1997 wheat crop and found that *Eurygaster austriaca seabrai* and *Aelia germari* were the most frequent species present in bug-damaged wheat. Nevertheless, scarce information has been reported about the protein modifications produced by these bugs.

Wheat attacked by *Nysius huttoni* showed high bug proteinase activity (Cressey 1987; Every et al 1990; Every 1992). Different studies by PAGE and SDS-PAGE demonstrated that proteases from *N. huttoni* specifically hydrolyze high molecular weight glutenin subunits (HMW-GS), yielding degradation products with an electrophoretic mobility similar to that of ω -gliadins. However, *N. huttoni* protease does not affect the gliadin fraction (Cressey and McStay 1987; Every et al 1990). In *Eurygaster* spp. damage, Yakovenko et al (1973) reported a similar selective degradation of the glutenin fraction, although the hydrolysis products had an electrophoretic mobility similar to that of the α -gliadins. Kozmina and Tvorogova (1973) described a reduction in the relative intensity of some bands in the electrophoretic pattern of total unreduced glutenin and gliadin fractions, and the appearance of two new bands with lower mobility in the gliadin pattern. Later, Sivri et al (1998) confirmed by electrophoresis studies that bug damage caused by *E. maura* affected the gliadins and the reduced glutenin fraction, where the HMW-GS were the most susceptible fraction to bug attack. Less information is available about the damage caused by *Aelia* spp.

Nevertheless, most protein studies describe the changes in the protein pattern without quantifying the extent of the bug damage in specific protein fractions. Sivri et al (1999) recently reported an attempt to quantify the effect of *E. maura* damage on the ratio of HMW-GS and LMW-GS among different wheat cultivars. These authors observed a high intercultivar variation when analyzing the ratio of HMW-GS and LMW-GS by RP-HPLC.

In Europe, bug damage is related to the disruption of polymeric glutenin proteins (HMW-GS, LMW-GS). Up to now, different approaches have been made to characterize those polymeric proteins although reduction of the proteins was always carried out. A better understanding of the effect of bug damage on wheat endosperm proteins might be reached by using size-exclusion high performance liquid chromatography (SE-HPLC) because this technique allows the characterization of wheat proteins without previous reduction and, hence, in their polymeric form (Gupta et al 1993; Larroque et al 1997).

High-performance capillary electrophoresis (CE) has emerged as a powerful technique in the characterization of cereal proteins (Bean et al 1998a; Bean and Lookhart 2000a,b, 2001). CE allows high-resolution electrophoretic separation of the cereal proteins, based on different charge density (free-zone capillary electrophoresis [FZCE]), size (SDS-CE) or isoelectric point (capillary isoelectric focusing [CIEF]).

The aim of the present study was to analyze the extent of *Aelia* spp. and *Eurygaster* spp. damage on wheat proteins, in terms of both amount and size distribution, by using the high-resolving techniques of SE-HPLC and CE to help understand the real mechanism of bug action. Different cultivars of Spanish bread wheats with a wide range of damage were studied.

MATERIALS AND METHODS

Several Spanish bread wheat cultivars (Marius, Soissons, Chamorro, and Astral) with and without bug damage were used to study the damage to the gluten proteins and the variability of damage on each cultivar due to insect attack. Wheat kernels (undamaged and damaged) from the 1999 crop of each cultivar were provided by the Asociacion Espanola de Tecnicos Cerealistas (AETC). Damaged samples were from the northern region of Spain, an endemic area of *Aelia* and *Eurygaster* infestation. In fact, a high number of insects was still present in the samples. Chemical reagents were purchased from Sigma (St. Louis, MO) and were of the highest purity.

Proteolytic Activity Assessment

A black spot surrounded by a pale area was visually observed in the damaged wheat. However, the extent of the damage was quantified by using the Chopin alveograph (Tripette et Renaud, Paris, France) following Approved Method 54-30 (AACC 2000). Berger et al (1974) found a good correlation between the deformation

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energy (W) change when the dough is allowed to stand at 25°C for 3 hr and the proteolytic degradation.

Sample Preparation

Alcohol-soluble proteins were obtained by vortexing 200 mg of whole meal with 1.0 mL of 50% 1-propanol for 5 min and centrifuging at 14,000 rpm for 2 min, as described Bean et al (1998b). These extracts contained albumins, globulins, gliadins, and soluble polymeric glutenins. The residues were washed twice with the same solution and centrifuged at 14,000 rpm for 2 min. After extraction, pellets were freeze-dried and then the protein content was analyzed by combustion method using a nitrogen determinator (Leco Corp., St. Joseph, MI) according to Approved Method 46-30 (AACC 2000). A conversion factor of 5.7 was used to transform nitrogen values to protein content.

A sequential extraction was made to obtain each class of proteins. Gliadins were extracted with 50% propan-1-ol after previous removal of albumins and globulins as reported in Bean et al (1998a). Polymeric proteins were extracted by using both nonreducing and reducing conditions. Nonreduced polymeric proteins were obtained by mixing the pellet with 1.0 mL of 1% SDS centrifuged at 14,000 rpm for 2 min. The reduced polymeric proteins were extracted with 1.0 mL of 50% propan-1-ol containing 1% DTT and then centrifuged at 14,000 rpm for 2 min. HMW-GS were prepared by acetone precipitation as described previously Bean and Lookhart (1998).

In incubated samples, whole meal (200 mg) was mixed with deionized water (0.5 mL) and kept under gentle magnetic stirring at 40°C for 3 hr, then the protein extraction was performed as described above.

Four different sets of samples from Marius, Soissons, Chamorro and Astral cultivars, with and without incubation, were run for each determination.

SE-HPLC Analysis

An Agilent 1100 Chromatograph was used for all the HPLC separations. Size-exclusion separation was performed by injecting 15 μ L of sample at 1.0 mL/min of acetonitrile (ACN)/water (50:50) containing 0.1% (w/v) TFA into a Phenomenex BioSep SEC-4000 column (Phenomenex, Torrance, CA). Protein elution was monitored at 220 nm.

Capillary Electrophoresis Analysis

Separations were made using a Beckman PACE 5510 instrument. Uncoated fused silica capillaries (Polymicro, Phoenix, AZ) of 50 μ m i.d. x 27 cm (20 cm L_D) were used for all separations.

Free-zone capillary electrophoresis (FZCE) was performed at the optimum separation conditions described by Bean and Lookhart (2000b): 50 mM iminodiacetic acid (IDA) containing 20% ACN and 0.05% hydroxypropyl methyl cellulose (HPMC), at 45°C and 30kV.

TABLE I
Proteolytic Activity of Bread Wheat Cultivars
Determined by Alveographic Test^a

Cultivar	Proteolytic Activity (%)
Marius	
Undamaged	0
Damaged	56
Soissons	
Undamaged	0
Damaged	53
Chamorro	
Undamaged	0
Damaged	42
Astral	
Undamaged	5
Damaged	61

^a Values are means of three replicates.

RESULTS AND DISCUSSION

Protein Size Modification by Bug-Damaged Attack

The proteolytic degradation of the wheat kernels used in this study was previously assessed by the alveographic method (Table I).

The alcohol-soluble proteins comprise albumins, globulins, gliadins, and some polymeric proteins. Typical SE-HPLC profiles are shown in Fig. 1. The first peak corresponds to the alcohol-soluble polymeric proteins, the second peak corresponds to gliadins, and the third peak corresponds to globulins and albumins with molecular mass <30 kDa (Larroque et al 1997). Instead of comparing the relative areas of the peaks for the different wheat cultivars, the quantitative method described by Bean et al (1998b) was used for this purpose. The protein content of the remaining pellet, after the extraction of the alcohol-soluble proteins, was determined to quantify the amount of the alcohol-insoluble proteins. The alcohol-soluble proteins were calculated by subtracting

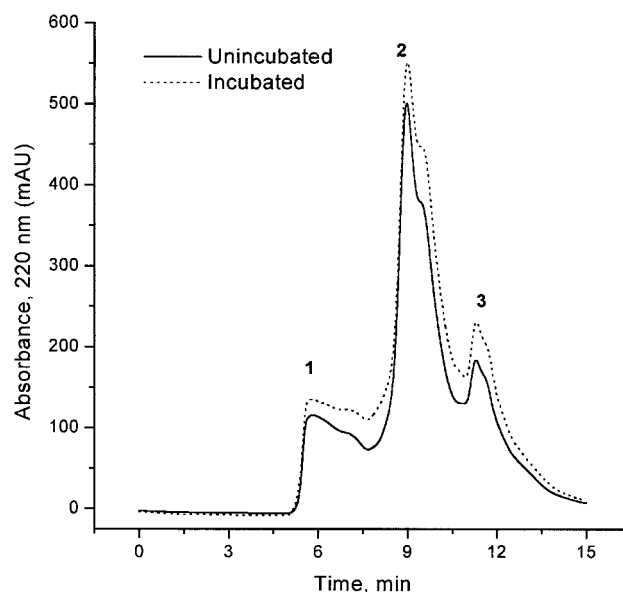


Fig. 1. Size-exclusion HPLC protein separation of alcohol-soluble extracts from wheat whole meal. Overlaid chromatograms of unincubated and incubated samples. Peaks: 1, alcohol-soluble polymeric proteins; 2, gliadins; 3, globulins and albumins with molecular mass <30 kDa.

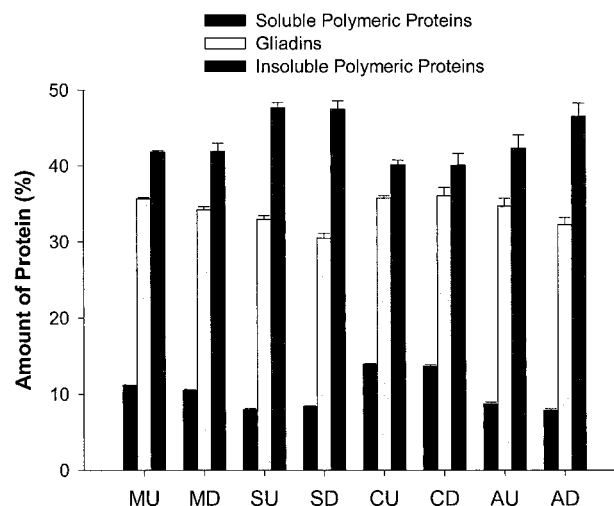


Fig. 2. Effect of bug damage on the amount of proteins in various protein fractions. Cultivars: M, Marius; S, Soissons; C, Chamorro; A, Astral. Samples: U, undamaged; D, damaged. Error bars indicate standard deviations ($n = 4$).

them from the total protein amount. The protein content of each SE-HPLC protein fraction was obtained by relating the protein content (mg) with the area of the peaks obtained by SE-HPLC. For comparative purposes, the percentage of each class of proteins was calculated. Figure 2 shows that, along with a high intercultivar variability, no significant differences in the alcohol-soluble polymeric protein content were found between undamaged and bug-damaged wheat. However, with the exception of Chamorro, undamaged wheats had slightly higher amounts of gliadins than damaged wheat. Sivri et al (1998) could not find a detectable effect of bug damage on the gliadins by using gel electrophoresis, but the quantitative SE-HPLC used in the present study showed differences. In Astral, the alcohol-insoluble polymeric proteins slightly increased with the bug attack. On the other hand, the total amount of alcohol-soluble proteins did not change with the bug attack, which means that the protein hydrolysis by bug proteinases led to changes in the protein size without modifying total alcohol-soluble protein content.

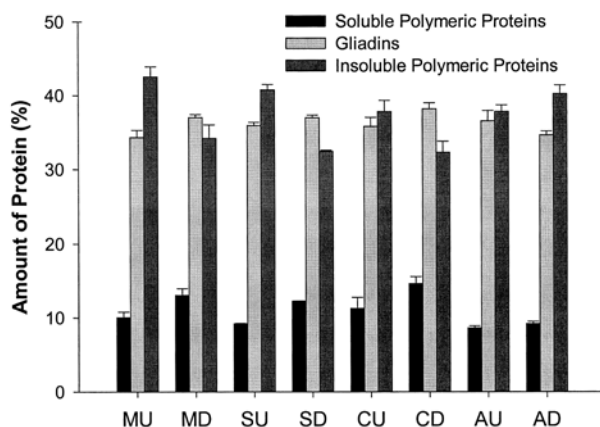


Fig. 3. Effect of bug attack on the amount of proteins in various protein fractions after incubation at 40°C for 3 hr. Cultivars: M, Marius; S, Soissons; C, Chamorro; A, Astral. Samples: U, undamaged; D, damaged. Error bars indicate standard deviations ($n = 4$).

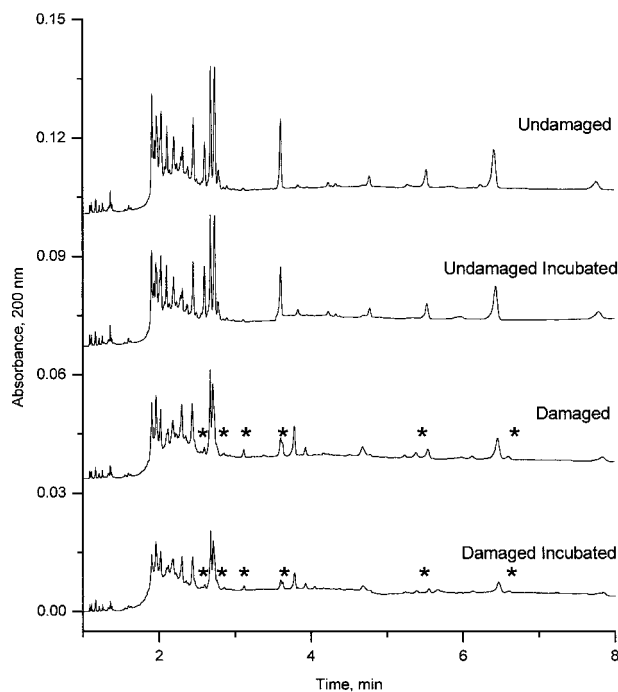


Fig. 4. Modification of the free-zone capillary electrophoresis (FZCE) pattern of gliadins from Soissons due to bug damage. Asterisks indicate differences among samples. Samples were pressure-injected (0.5 psi) for 2 sec.

The SE-HPLC gliadin profile of incubated samples showed a large modification (Fig. 1). These differences were observed in the undamaged and damaged samples. Although the extent of the modification was varied, it was more pronounced in the damaged samples than in the undamaged ones (Fig. 3).

A decrease in the amount of alcohol-insoluble polymeric proteins was observed in the incubated undamaged samples from Soissons and Astral, but no change was observed in undamaged Marius and Chamorro samples. The products derived from the hydrolysis of the alcohol-insoluble polymeric proteins eluted with the gliadins, yielding an increase of this fraction. The modification of the protein profile observed in the undamaged samples after incubation might be due to endogenous proteolytic enzymes, although no detectable activity was measured in Soissons by the alveographic method.

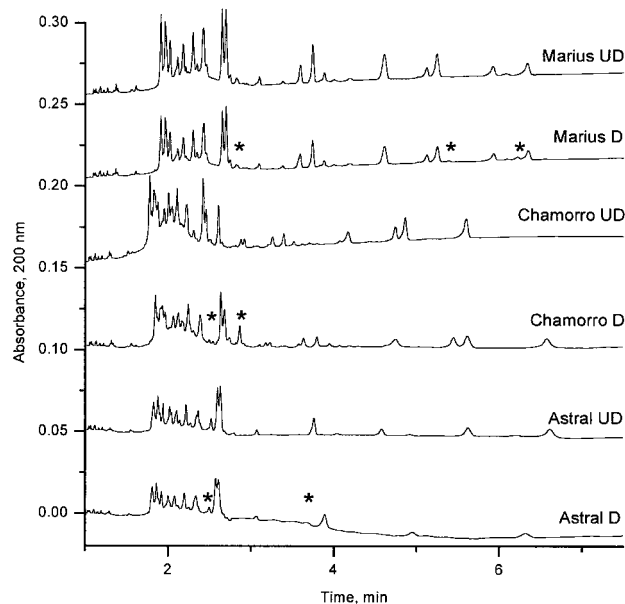


Fig. 5. Modification of the free-zone capillary electrophoresis (FZCE) pattern of gliadins from different cultivars due to bug damage. UD, undamaged wheat; D, damaged wheat. Asterisks indicate differences among samples.

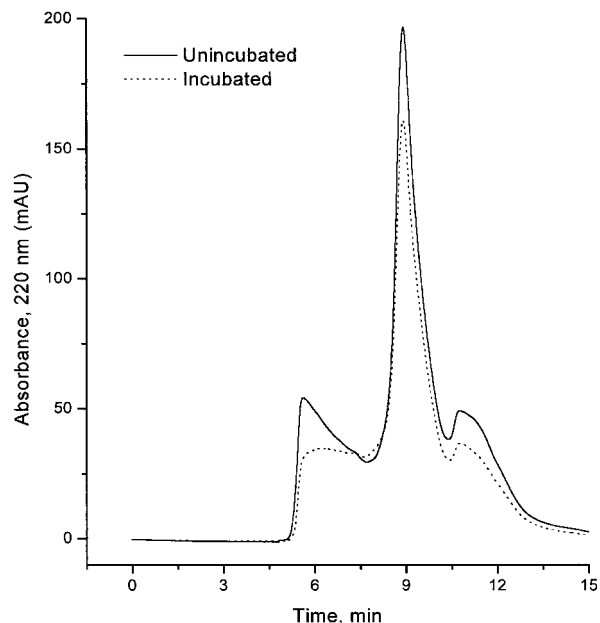


Fig. 6. Size-exclusion HPLC separation of gliadin fraction after removal of albumins and globulins.

A severe decrease of the alcohol-insoluble polymeric proteins along with an increase of the alcohol-soluble polymeric proteins and gliadins were observed after incubation of the damaged samples. Nightingale et al (1999) obtained analogous results when they studied the effect of *Fusarium* spp. on wheat storage proteins by SE-HPLC. In that study, they also reported a large decrease of the HMW proteins concomitant with a redistribution of the proteins to LMW.

The intensity of the hydrolysis varied among cultivars, with Soissons being the most affected, although similar degradation activities were measured by the alveograph test. It may be that the protein structure of Soissons is more susceptible to bug proteinase hydrolysis. Similar findings were obtained by Every et al (1998) and Sivri et al (1999), who observed different cultivar susceptibilities to bug damage. They attributed this variability to intrinsic differences in glutenin structure. The changes observed in the alcohol-soluble proteins supported the results of Kretovich (1944), where bug infection increases the protein solubility in alcohol and in water.

Effect of Bug Damage on the Gliadin Fraction

To determine the specific effect of the bug damage on the gliadins, these proteins were extracted following the removal of albumins and globulins and analyzed by FZCE. Damaged wheat from all the cultivars tested showed a decrease in a peak at the γ gliadin mobilities, and new peaks appeared at the lowest mobilities in the ω gliadin range. Those results could be due to the hydrolysis of polymeric proteins, or insect feeding on immature kernels and causing interference with grain development and, in consequence, the laying down of specific proteins. The extent of the difference between undamaged and damaged wheat was greatly dependent on the cultivar. Soissons showed the greatest modification between undamaged and damaged samples, where some new peaks in the ω gliadin mobility zone were noted (Fig. 4). In the other cultivars, electrophoregrams of the gliadins also revealed differences between undamaged and damaged samples in the γ and ω gliadin mobility range (Fig. 5).

Incubation of damaged samples caused a considerable decrease in the intensities of all the gliadin peaks without changing the mobilities. Only a slight decrease in intensity was observed in the

undamaged samples. However, a very pronounced decrease was observed in the damaged samples, which contained smaller amounts of gliadin proteins (Fig. 4). These results appeared to be contradictory because SE-HPLC and protein quantification had shown that gliadin content increased with incubation. To explain those differences, the isolated gliadin fractions were analyzed by SE-HPLC. Once again, the relative amount of gliadins (peak 2) decreased after incubation (Fig. 6). This difference can be explained by a size overlapping of the hydrolysis products produced by bug proteinase and the gliadins, which was not observed when gliadins were extracted after removal of the salt-soluble compounds. In fact, an increase of the salt-soluble compounds was observed after incubation when those fractions were analyzed by SE-HPLC (results not shown).

Changes in intensities and mobilities have been described in the bug-damaged wheat caused by *E. maura*, but only after incubation (Sivri et al 1998). In this study, the effect of bug damage on gliadins was readily observed before incubation by using FZCE.

By using FZCE, some new peaks in the ω gliadin region were detected as well as a decrease of all gliadin peaks, the later being accentuated by the incubation. From these results, it is evident that gliadins are affected by bug damage, but the decrease in all gliadin proteins might be explained by a dilution effect due to the increase in the alcohol- and salt-soluble protein content or the presence of a nonspecific proteinase in bug insects.

In New Zealand, bug damage caused by *N. huttoni* does not modify the gliadin patterns but hydrolysis products from polymeric proteins with electrophoretic mobilities similar to ω gliadins have been detected (Cressey and McStay 1987; Swallow and Cressey 1987; Every et al 1990).

Effect of Bug Damage on the Glutenin Fraction

Glutenin proteins extracted under nonreducing conditions were analyzed by SE-HPLC. No differences could be attributed to bug attack because a high variability was observed between cultivars (results not shown). Comparison of the total area beneath the chromatograms showed a slight decrease of the amount of extracted glutenins in the incubated samples. This effect was observed in the undamaged and damaged wheat. Therefore, it should be more ascribed to en-

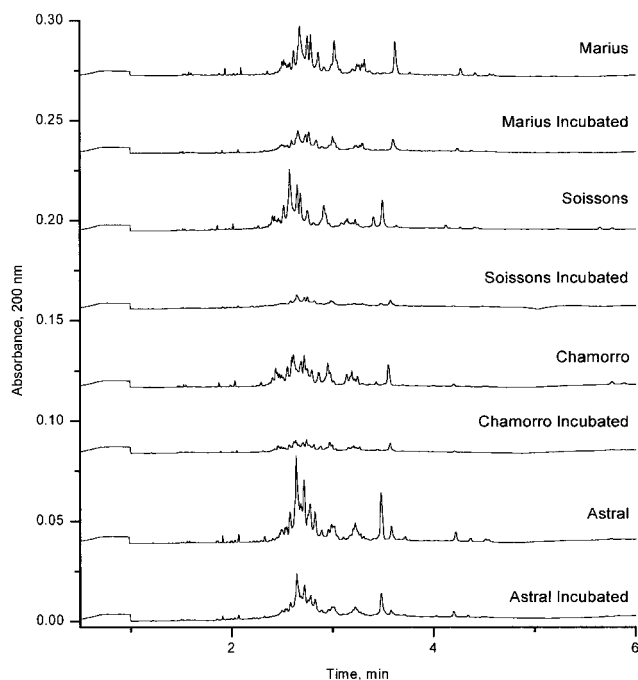


Fig. 7. Free-zone capillary electrophoresis (FZCE) of low molecular weight glutenin fractions from different bug-damaged cultivars. Samples were pressure-injected (0.5 psi) for 4 sec.

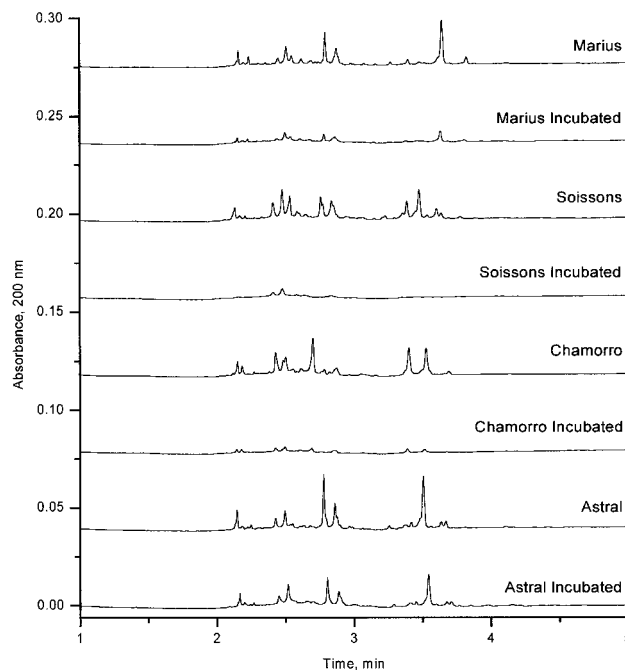


Fig. 8. Free-zone capillary electrophoresis (FZCE) of high molecular weight glutenin fractions from different bug-damaged cultivars. Samples were pressure-injected (0.5 psi) for 4 sec.

dogenous protease activity rather than bug proteinase effect. Glutenins extracted under reducing conditions were analyzed by FZCE. Comparison of the FZCE profiles of undamaged (unincubated and incubated) and damaged samples (unincubated) revealed no qualitative differences. However, a decrease of all the glutenin peaks was observed in the incubated damaged samples (results not shown).

It has been reported that bug proteinases hydrolyze glutenins, and within them they specifically degrade HMW-GS (Cressey and McStay 1987; Sivri et al 1998, 1999). To identify the possible specificity of the bug proteinase, HMW-GS and LMW-GS were analyzed by FZCE. The electrophoregrams of the LMW-GS from undamaged samples did not show changes, even after incubation.

However, comparison of the electrophoregrams of LMW-GS from damaged samples showed a large decrease of the peaks after incubation, which was barely detected in Soissons and Chamorro (Fig. 7).

No differences were observed between unincubated undamaged, unincubated damaged HMW-GS, and incubated undamaged samples. HMW-GS electrophoregrams from incubated damaged samples showed a profound decrease of all the proteins and, again, they were hardly visible in Soissons and Chamorro (Fig. 8). Astral was the least affected by the bug attack; it showed the lowest modification of the HMW-GS and LMW-GS fractions. Those fractions (HMW-GS and LMW-GS) were also analyzed by using SDS-CE to determine a possible size change, but no differences were detected in any of them (results not shown). These findings indicate that the proteinases from Spanish bug pests hydrolyze glutenins specifically, but they do not have any specificity for HMW-GS or LMW-GS.

These results are similar to those obtained by Sivri et al (1998), who reported a decreased of the LMW-GS and HMW-GS in damaged wheat caused by *Eurygaster*, but marked differences were observed among cultivars. Conversely, Every et al (1990, 1998) only described a decrease of the HMW-GS in wheat damaged by *Nyctelia*.

CONCLUSIONS

Protein modification promoted by attack of bug pests can be quantified by using SE-HPLC combined with protein quantification. Some differences can be detected in the unincubated undamaged and damaged wheat, although they are largely dependent on the cultivar. When samples were incubated, bug proteinases produced profound decreases in the alcohol-insoluble polymeric proteins concomitant with an increase of the alcohol-soluble polymeric proteins, gliadins, and albumins and globulins. Free-zone capillary electrophoregrams of each protein fraction allow characterization of the protein disruption caused by bug proteinase and, in consequence, FZCE might be a useful tool for rapid determination of bug attack.

The high intercultivar variability suggests that further studies are needed to determine the intrinsic resistance of some cultivars to the bug proteinase attack.

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