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Prolamin proteins alteration in durum wheat by species of the genus *Eurygaster* and *Aelia* (Insecta, Hemiptera)**

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Abstract

Wheat bugs are widely distributed in various areas of Europe, Asia and North Africa. Species belonging to the genus *Eurygaster* and *Aelia* pierce wheat kernels affecting protein quality. The effects of these insects' feeding activity have been studied mainly in bread wheat (*Triticum aestivum* L.). This study provides information on the degradation of prolamin proteins (glutenins and gliadins) of bug-damaged durum wheat (*Triticum turgidum* L. var *durum*) in six cultivars grown in Sardinia (Italy). Samples of whole flour mixture of 70% sound wheat and 30% damaged wheat were hydrated and incubated at two temperatures (45 and 4°C), for different periods of time (0, 1 and 3 h). Glutenin and gliadin content was analysed using free zone capillary electrophoresis. The presence of bug-damaged kernels had influence on the quality of durum wheat proteins. Glutenins were rapidly degraded independently to incubation temperature. Gliadin degradation, however, took place with dependence on temperature and incubation time. Therefore glutenin degradation was possibly not due solely to the activity of proteolytic enzymes but also to some other as yet unknown factor linked to wheat bugs' feeding activity.

Additional key words: gliadin, glutenin, protein quality, *Triticum turgidum* L. var *durum,* wheat bugs.

Resumen

Alteración de las prolaminas en trigo duro por especies del género *Eurygaster* y *Aelia* (Insecta, Hemiptera)

Los chinches de los cereales tienen una amplia distribución en diversas áreas de Europa, Asia y Norte de África. Especies pertenecientes a los géneros *Eurygaster* y *Aelia* se alimentan picando los granos de trigo afectando a la calidad de las proteínas. Esta alteración se ha estudiado principalmente en trigo blando (*Triticum aestivum* L.). El presente estudio aporta información sobre la degradación de las prolaminas (gluteninas y gliadinas) del trigo duro (*Triticum turgidum* L. var *durum*) dañado por los chinches de los cereales en seis variedades cultivadas en Cerdeña (Italia). Muestras de harina integral, con un 70% de trigo sano y un 30% de trigo dañado se incubaron a dos temperaturas (45 y 4°C), y a diferentes tiempos (0, 1 y 3 h). El contenido de las gluteninas y gliadinas se analizó mediante electroforesis capilar zonal. La presencia de granos picados por los chinches de los cereales influyó en la calidad de las proteínas del trigo duro. Las gluteninas se degradaron rápidamente, con independencia de la temperatura de incubación. En cambio, la degradación de las gliadinas resultó ser dependiente de la temperatura y del tiempo. Ante estos resultados, cabe plantearse la posibilidad de que la degradación de las gluteninas no sea debida sólo a la actividad de las proteasas sino también a algún otro factor ligado a la actividad alimenticia de los chinches de los cereales, todavía por determinar.

Palabras clave adicionales: calidad de las proteínas, chinches de los cereales, gliadinas, gluteninas, *Triticum turgidum* L. var *durum*.

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Abbreviations used: A-PAGE (acid polyacrylamide gel electrophoresis), DTT (dithiothreitol), FZCE (free zone capillary electrophoresis), HMW-GS (high molecular weight glutenin subunits), IDA (iminodiacetic acid), LMW-GS (low molecular weight glutenin subunits), RP-HPLC (reverse phase-high performance liquid chromatography).

Introduction

Wheat (*Triticum* spp.) is one of the three most important crops in relation to cultivated area (approximately 215 million hectares) and total global production (approximately 600 million tonnes) (FAOSTAT, 2008). The unique properties of wheat flour and dough enable the production of a wide range of products such as various types of bread, pasta, cakes and biscuits (Shewry *et al*., 1997).

Durum wheat (*Triticum turgidum* L. var *durum*) is a very important crop in Italy, the production in 2007 was approximately 4.1 million tonnes (ISTAT, 2008), around 15% of the world production. Durum wheat is used mainly for the manufacture of pasta as well as for baking traditional types of bread (Quaglia, 1988). The semolina obtained from durum wheat is the preferred prime matter for the manufacture of superior quality pasta (Feillet and Dexter, 1996).

Different factors can be detrimental to wheat crops, agronomical factors, environmental conditions, pests, diseases, etc., which can result in loss of quantity and quality of wheat, semolina or flour. Wheat bugs of the genus *Eurygaster* (Hemiptera, Heteroptera, Fam. Scutelleridae) and *Aelia* (Hemiptera, Heteroptera, Fam. Pentatomidae), also known as Sunn pest, affect both aforementioned aspects.

Eurygaster and *Aelia* wheat bugs are widely distributed in various areas of Europe, Asia and North Africa (Paulian and Popov, 1980) and an estimate of more than 15 million hectares of cereals (mainly wheat and barley) are infested annually in Syria, Iraq, Iran, Turkey, Afghanistan and Lebanon, as well as in Central Asia and the Caucasus, Bulgaria and Romania (El Bouhssini *et al*., 2002). There are long term studies focussed on integrated pest control (El Bouhssini *et al*., 2002; Parker *et al*., 2003) and on the effects on protein alteration caused by the salivary residues left in the kernels by the insects' feeding (Kretovich, 1944; Karababa and Ozan, 1998; Sivri *et al*., 1998, 1999, 2004; Hariri *et al*., 2000; Aja *et al*., 2004; Caballero, 2005; Ozderen *et al*., 2008; Werteker and Kramreither, 2008). Effects on the alteration of starch granules have been reported for *Nysius* spp. (Hemiptera, Heteroptera, Fam. Lygaeidae), a wheat bug present in Australia and New Zealand (Every *et al*., 1990; Lorenz and Meredith, 1998) but amylase activity is not involved in the wheat damage caused by *Aelia* spp. and *Eurygaster* spp. (Rosell *et al*., 2002a).

Both nymph and adult *Eurygaster* and *Aelia* wheat bugs insert their piercing-sucking mouthparts in the wheat kernels and extract the substances within. In order to facilitate the suction of the nutritional elements of the endosperm, the kernels are digested externally by injecting saliva rich in proteolytic enzymes (Sivri *et al*., 1998) and amylases (Kazzazi *et al*., 2005). Pierced kernels in the field usually continue to mature. When ripe, bug-damaged kernels present a whitish opaque spot and sometimes also a small black dot where the kernel was pierced (Hariri *et al*., 2000).

There is a general agreement that durum wheat protein content is the primary factor influencing rheological properties and pasta quality (D'Egidio *et al*., 1990; Novaro *et al*., 1993; Feillet and Dexter, 1996). Protein content and amino acid composition of wheat vary depending mainly on genotype and agro-climatic conditions (López-Bellido *et al*., 1998; Rharrabti *et al*., 2003; Dupont *et al*., 2006).

Gluten proteins are to a considerable extent responsible for the functional properties of flour. Wheat gluten consists of more than 50 protein components (Shewry *et al*., 1987) that have been traditionally classified into two groups, gliadins and glutenins (Wieser, 2007). Gliadins are soluble in aqueous alcohols (60-70% ethanol, 50% 1-propanol) and are present as monomeric proteins that lack inter-chain disulphide bonds, presenting instead intra-chain disulphide bonds (Shewry *et al*., 1997). Glutenins are insoluble in aqueous alcohols and consist of protein subunits present in polymers stabilised by inter-chain disulphide bonds. A reduction of these bonds results in subunits which are soluble in alcohol/water mixtures. Rheological properties of dough depend on both protein fractions (Shewry *et al*., 1997).

Although the effects of wheat bugs' feeding activity on bread wheat have been largely studied (Karababa and Ozan, 1998; Sivri *et al*., 1999, 2004; Hariri *et al*., 2000; Aja *et al*., 2004; Vaccino *et al*., 2006; Werteker and Kramreither, 2008) there are not many works regarding durum wheat and other species of the genus *Triticum*. There are various studies on the percentage of bug-damaged kernels necessary in order to seriously affect the kernel, flour, dough or bread quality parameters. Wheat samples which had more than 5% bug-damaged kernels changed their physicochemical properties and showed significantly lower quality (Karababa and Ozan, 1998; Hariri *et al*., 2000). The aim of this study was to investigate how the feeding activity of cereal bugs of the genus *Eurygaster* and/or *Aelia* affects glutenins and gliadins in durum wheat at different incubation conditions concerning temperature (45 and 4°C) and time (0, 1 and 3 h).

Material and methods

Wheat samples

Six cultivars (Karalis, Asdrubal, Claudio, Rusticano, Colosseo and Canyon) of durum wheat grown in extensive commercial fields near Villamar in the south-centre of Sardinia island (Italy) were tested. Those samples were provided by Laore, the Regional Agency of Sardinia for the Development of Agriculture. The samples showed a percentage of damaged kernels ranging from 1.6 to 4.1%. In each sample, bug-damaged kernels, characterized by a typical whitish opaque spot and also very often a black dot, were separated visually from sound kernels. Two series of sub-samples were prepared for each cultivar in order to carry out the protein analysis. One of the sub-samples consisted of only sound kernels without visible defects; the other sub-samples consisted of medium level bug-damaged kernels —*i.e.,* with a damage between 1/3 and 2/3 of the total grain surface—, lacking other visible defects. The sound kernels were ground using a Perten 3100 laboratory grinder to obtain wholemeal flour, whereas a Culloti laboratory grinder was used for the damaged kernels because of their scarcity. Both mills had a 0.8 mm sieve.

Protein alteration test, capillary electrophoresis analysis

For each cultivar, two types of samples of whole flour were worked out: on one hand, samples of sound flour (named «sound wheat», used to perform a control or stability test), and on the other hand samples of a blend of 70% sound flour and 30% bug-damaged flour (named «damaged wheat» used to perform a degradation test).

All samples, after hydration with deionised water, were tested using different incubation times (0 h = unincubated, 1 h = 1 hour and 3 h = 3 hours) and temperatures (45 and 4°C). Temperatures tested by authors in studies on bread wheat usually range from 37°C to 45°C (Sivri *et al*., 1998, 1999; Aja *et al*., 2004), whereas 4°C is a temperature that has not been tested previously.

The proportions of the blend of wheat, 70% sound wheat and 30% damaged wheat, as well as incubation conditions at 45°C were selected in order to provide a sufficient degree of protein degradation and also because they had already been tested on bread wheat (Sivri *et al*., 1999) therefore providing a comparative frame to our results. Incubation at 4°C was chosen to assess if degradation can be blocked. All the assays were carried out at least in duplicate.

A sequential protein extraction (albumins, globulins, gliadins and glutenins) was carried out on each sample following the protocol described by Bean and Lookhart (1998). For each cultivar, a sample of 100 mg of sound wheat or damaged wheat was hydrated with 1 mL of deionised water for incubation or to start off protein fractioning, specifically albumins. After stirring for 5 min the sample was centrifuged at 14,000 rpm for 5 min; the supernatant was discarded and the pellet resuspended using 1 mL of extraction buffer (50 mM Tris HCl, 50 mM KCl, 5 mM EDTA, at pH 7.8) so as to discard globulins; this operation was repeated twice, always stirring for 5 min and centrifuging at 14,000 rpm. The pellet was then resuspended using 1 mL of 50% 1-propanol (v/v) in order to extract the gliadins. The pellet was cleaned yet again with 1 mL of 50% 1-propanol. Finally, the glutenins were obtained by resuspending and stirring the pellet with 50% 1-propanol + 1% DTT (v/v) for 30 min and then centrifuging at 14,000 rpm for 5 min. For the samples incubated at 4°C, this temperature was maintained up to albumin and globulin extraction to avoid possible activity of the proteases on the gliadin and glutenin fraction. Gliadin and glutenin extracts were filtered at 45 µm.

Analysis of the different gliadin and glutenin subunits was carried out using FZCE. The process followed was similar to the method described by Bean and Lookhart (2000), but adjusted to a Hewlett-Packard CE and using a silex capillary tube (34 cm Polymicro Phoenix AZ, 25.5 cm L_D, 50 µm i.d.) 50 mba × 8 s for injection of the samples, an IDA buffer (50 mM IDA, 20% acetonitrile and 0.05% hydroxypropylmethylcellulose) at 30 kV and 45°C.

Gliadin and glutenin alteration was assessed by comparing the electropherograms obtained from the sound wheat and damaged wheat in the incubation conditions described. The alterations in the total area (total quantity of gliadins or glutenins expressed in mAU*s) of the electropherograms were considered to be signs of protein alteration.

Statistical analysis

The results express the mean values obtained from two repeated trials. Factorial analysis of variance of glutenins and gliadins was carried out using the factors cultivar, temperature and incubation time. The results

were analyzed using the Tukey method. The statistical analysis of data was carried out using the SAS software. A level of significance of 0.05 ($P < 0.05$) was used throughout the study.

Results

Glutenins

The stability test proved that glutenins from sound wheat were stable, with no significant differences in the amount of glutenins between the sound wheat unincubated and the sound wheat incubated for 3 h at 45°C. This allowed to use the mean value of duplicated sample of unincubated sound wheat as a reference value in order to evaluate degradation; an example of this can be observed in the stability test of cv. Karalis, shown by the equivalent electropherogram area in Figure 1a-1b. The degradation test proved (Fig. 1c-1d) that the glutenins of the damaged wheat, on the other hand, were degraded rapidly even in unincubated samples (Table 1, Fig. 2), with an average loss compared to the sound wheat samples of –65%. These differences were significant in all wheat cultivars when compared to the sound wheat (Fig. 2). Degradation was slightly higher when the damaged wheat samples were incubated (Table 1). The average degradation for 1 h was –79% at 4°C and –78% at 45°C. The average degradation for 3 h at both 4°C and at 45°C was –86% (Table 1). Differences between sound wheat samples and incubated (1 h and 3 h) damaged wheat samples were significant for all cultivars (Fig. 2). Not all samples showed significant differences between incubation at 1 h or 3 h at the same temperature (Fig. 2). For each cultivar, there were no significant differences between incubation at 45°C and at 4°C for the same incubation time, with the exception of Rusticano and Asdrubal cultivars incubated for 1 h and 3 h respectively (Table 1). Thus it can be stated, with some exceptions, that glutenins were degraded to the same degree at 45°C as at 4°C.

Gliadins

The stability test proved that gliadins from sound wheat were stable, with no significant differences in the amount of gliadins between the sound wheat

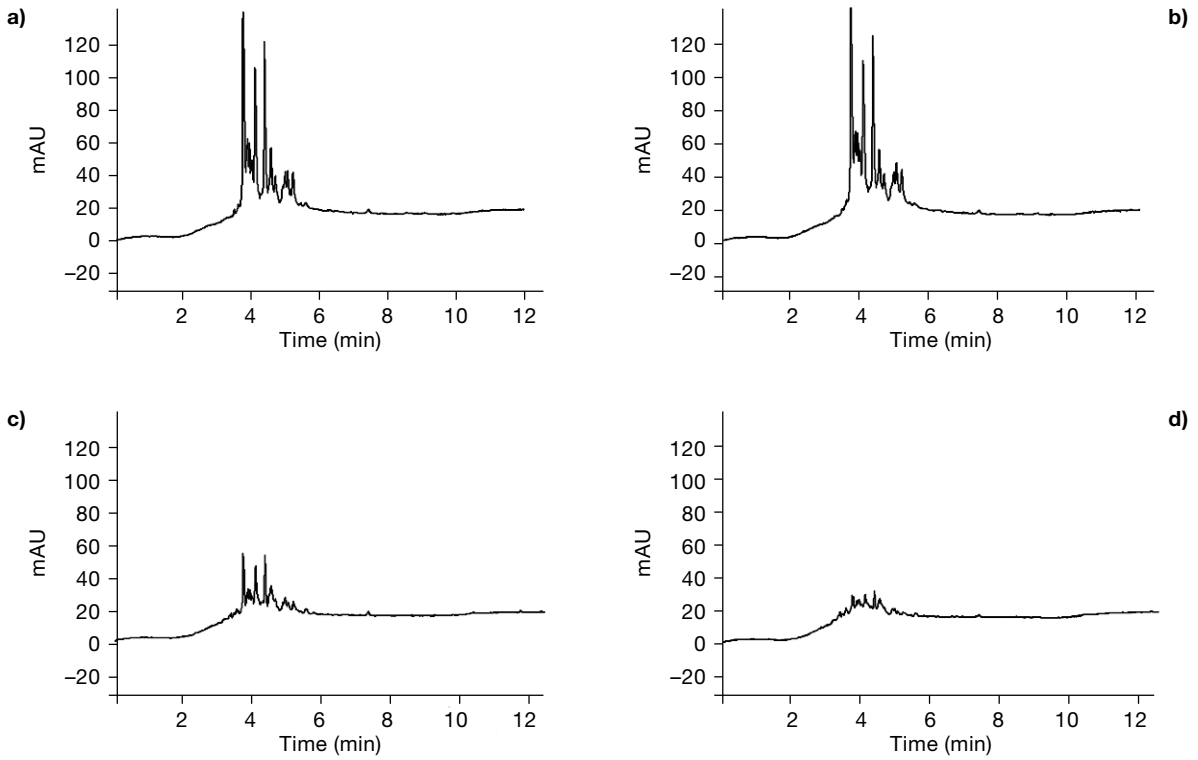

Figure 1. FZCE electropherograms of cv. Karalis glutenins (50% 1-propanol + 1% DTT) from sound wheat unincubated (a) and incubated at 45°C 3 h (b), and damaged wheat incubated at 45°C for 1 h (c) and 3 h (d).

Table 1. Quantification of the alteration of total glutenins and gliadins of the damaged wheat at different temperatures (45°C, 4°C) and different incubation times (0 h, 1 h and 3 h) compared to sound wheat, expressed as loss (–) or gain (+) of area, in percentage and mAU*s (total area). For each cultivar, data within columns followed by the same letter are not significantly different ($P < 0.05$).

	Karalis		Asdrubal		Claudio		Rusticano		Colosseo		Canyon		Mean*
	%	mAU*s	%	mAU*s	%	mAU*s	%	mAU*s	%	mAU*s	%	mAU*s	%
Total glutenins													
Sound wheat		3,718^{a}		4,250^{a}		3,822^{a}		3,950^{a}		3,823^{a}		4,418^{a}	
Damaged wheat 0 h 4°C	–63	–2,345^{b}	–60	–2,554^{b}	–65	–2,472^{b}	–58	–2,342^{b}	–73	–2,795^{b}	–69	–3,047^{b}	–65 ± 5.5
Damaged wheat 1 h 45°C	–75	–2,779^{c}	–73	–3,084^{c}	–82	–3,143^{c}	–66	–2,611^{c}	–84	–3,198^{c}	–87	–3,853^{cd}	–78 ± 8.0
Damaged wheat 1 h 4°C	–79	–2,920^{cd}	–70	–2,992^{c}	–85	–3,234^{cd}	–74	–2,926^{d}	–84	–3,224^{c}	–84	–3,704^{d}	–79 ± 6.0
Damaged wheat 3 h 45°C	–80	–2,987^{cd}	–86	–3,641^{d}	–88	–3,346^{cd}	–80	–3,157^{de}	–91	–3,489^{d}	–91	–4,009^{c}	–86 ± 4.9
Damaged wheat 3 h 4°C	–82	–3,053^{d}	–81	–3,454^{e}	–90	–3,451^{d}	–83	–3,271^{e}	–91	–3,492^{d}	–91	–4,008^{c}	–86 ± 4.8
Total gliadins													
Sound wheat		2,958^{ab}		3,511^{a}		3,179^{a}		3,242^{ab}		3,149^{ac}		3,862^{a}	
Damaged wheat 0 h 4°C	+4	+114^{a}	–11	–386^{ac}	+5	+175^{a}	+5	+167^{a}	+30	+943^{b}	–9	–330^{a}	+4 ± 14.6
Damaged wheat 1 h 45°C	+11	+334^{a}	–18	–637^{abc}	–32	–1,023^{b}	–22	–718^{b}	–18	–559^{a}	–38	–1,485^{b}	–20 ± 17.2
Damaged wheat 1 h 4°C	–9	–264^{ab}	–16	–557^{abc}	–6	–179^{a}	+8	+250^{a}	+17	+551^{bc}	–18	–689^{ac}	–4 ± 13.8
Damaged wheat 3 h 45°C	–56	–1,652^{c}	–41	–1,438^{b}	–55	–1,763^{c}	–50	–1,624^{c}	–70	–2,193^{d}	–66	–2,563^{d}	–56 ± 10.5
Damaged wheat 3 h 4°C	–24	–718^{b}	–31	–1,098^{c}	–6	–205^{a}	–5	–160^{ab}	–11	–332^{a}	–35	–1,342^{bc}	–19 ± 13.1

* Mean: mean of the alteration of the six cultivars ± standard deviation.

unincubated and the sound wheat incubated for 3 h at 45°C. This allowed to use the mean value of the duplicated sample of unincubated sound wheat as a reference value in order to evaluate degradation; an example of this can be observed in the stability test of cv. Karalis shown by the equivalent electropherogram area in Figure 3a and 3b. The behaviour of gliadins in the damaged wheat samples differed strongly from that of glutenins. It was found that, in contrast to that of glutenins, degradation of gliadins was much lower and was time and temperature dependent (Table 1, Figs. 3c-3d and 4). Most of the unincubated (0 h) damaged wheat samples showed a total gliadin area increase (Table 1, Fig. 4), presumably due to the depolymerised glutenins. These increments ranged from +4% to +30% compared to the sound flour, with the exception of the cvs. Asdrubal and Canyon that lost –11% and –9%, respectively (Table 1). These differences, however, compared to the sound wheat, were only significant for the Colosseo cultivar (Fig. 4). For the damaged wheat samples incubated for 1 h at 45°C degradation ranged from –18% to –38% with the exception of the cv. Karalis where an increase of +11% was registered (Table 1). The differences were only significant, compared to the sound wheat, in the cvs. Claudio and Canyon (Fig. 4). There were minor variations in the damaged wheat samples incubated for 1 h at 4°C (from +17% to –18%, Table 1) and no significant differences when compared to the sound wheat (Fig. 4). However increases in gliadin degradation were significant for all cultivars,

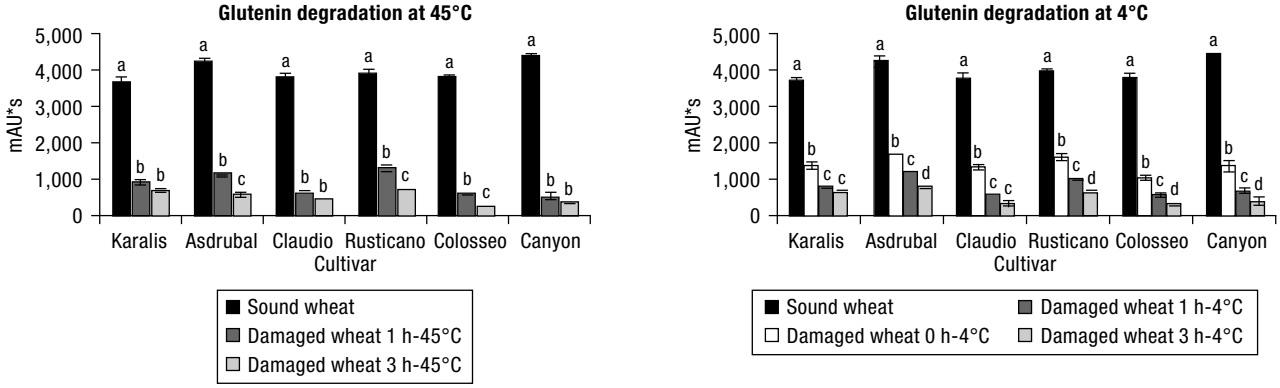

Figure 2. Results for glutenin degradation, comparing sound wheat and damaged wheat, in six durum wheat cultivars, at different temperatures (45 and 4°C) and different incubation times (0 h, 1 h and 3 h) expressed as total area in mAU*s. Bars describe standard deviation. For each cultivar at 45°C or at 4°C, the same letter indicates not significant differences (Tukey test, $P < 0.05$).

compared to the sound wheat, when damaged wheat samples were incubated for 3 h at 45°C (Fig. 4) In this case the average degradation percentage was –56% of the total gliadins (Table 1). Degradation of gliadins incubated for 3 h at 4°C, with an average degradation of –19%, was much lower than in samples incubated for 3 h at 45°C (Table 1). For each cultivar, there were significant differences between incubation at 45°C and at 4°C for 3 h. Above all, it can be stated that gliadins were not degraded to the same degree at 45°C as at 4°C.

Discussion

The previous results show that wheat bugs have a strong influence on durum wheat proteins determining quality loss. Total glutenin and gliadin alteration, due to the presence of bug-damaged kernels, was quantified and results were similar to those described in bread wheat (analysis perfomed only at high temperature). Glutenin results were very much alike to those obtained in bread wheat (Sivri *et al.*, 1998, 1999;

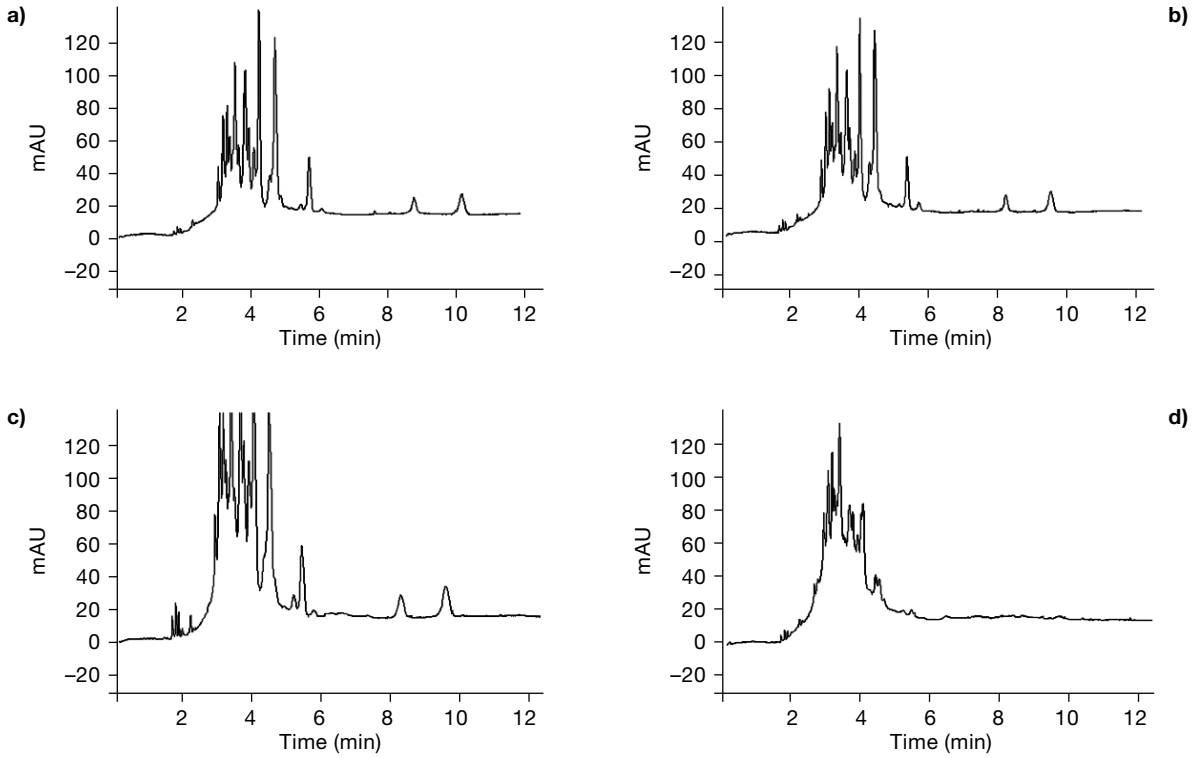

Figure 3. FZCE electropherograms of cv. Karalis gliadins (50% 1-propanol + 1% DTT) from sound wheat unincubated (a) and incubated at 45°C for 3 h (b), and damaged wheat incubated at 45°C for 1 h (c) and 3 h (d).

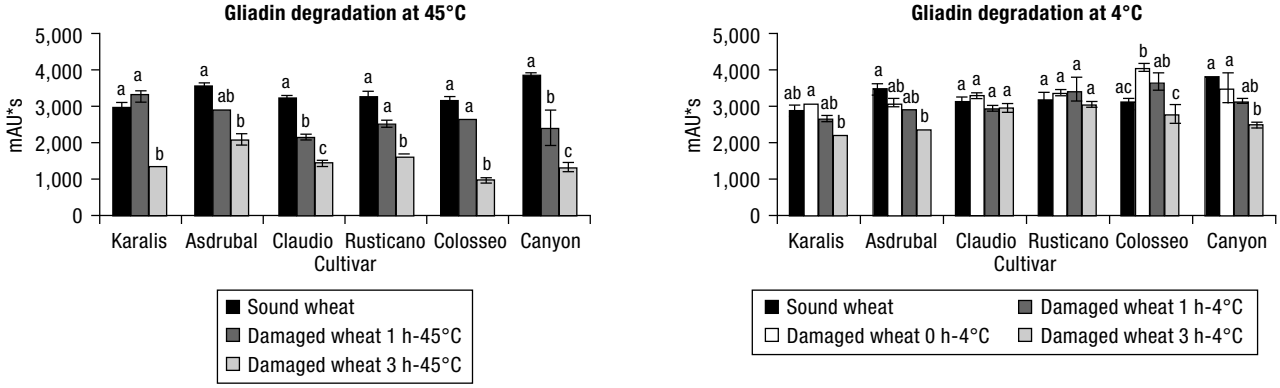

Figure 4. Results for gliadin degradation, comparing sound wheat and damaged wheat, in six durum wheat cultivars, at different temperatures (45 and 4°C) and different incubation times (0 h, 1 h and 3 h) expressed as total area in mAU*s. Bars describe standard deviation. For each cultivars at 45°C or 4°C, the same letter indicates not significant differences (Tukey test $P < 0.05$).

Rosell *et al*., 2002b). In wheat cultivated in Turkey and manually infested by *E. maura* (L.) bugs, it was seen that after 30 min incubation, more than 80% glutenin was degraded, and increase of degradation was very low 1 h and 2 h after incubation (Sivri *et al*., 1999). In that research, the proportions (2:1) of sound and damaged wheat in the blend were similar to those used in the present study, temperature of incubation was 37°C, and determination of reduced glutenins was done by (RP-HPLC). In bug-damaged bread wheat cultivated in Spain, a decrease of the glutenin fractions HMW-GS and LMW-GS determined with FZCE was also reported (Rosell *et al*., 2002b). Regarding bug-damaged wheat, less information is available on gliadins than on glutenins. The initial increase in the area of gliadins observed in this study, probably due to the presence of glutenin degradation products with a higher electrophoretic mobility, was also described in bug-damaged bread wheat, as in the case of Spanish bread wheat samples and using FZCE (Rosell *et al*., 2002b). In gliadins from bread wheat from Turkey incubated at 37°C, and analysed by A-PAGE, a decrease was also stated, concerning some new bands as well as the original gliadin bands; as in the case of durum wheat, the changes were more obvious with increasing incubation times and most of the gliadin bands were lost after 120 or 240 min of incubation (Sivri *et al*., 1998).

In bug-damaged bread wheat a marked increase of free thiol groups during initial incubation was described (Pérez *et al*., 2005). Results in durum wheat damaged by wheat bugs indicate that the alteration of the gliadins and glutenins initially begins with a fast depolymerisation of glutenins, independently of temperature, presumably due to a reduction of inter-chain disulphide bonds. The alteration continues, depending on the temperature, showing a general and specific degradation of gliadins and solubilised glutenins. Therefore, degradation of glutenins was possibly due not only to the action of protease enzymes, but also to another not yet determined salivary agent(s), that would act as a reductor. These durum wheat results could explain the modification of pasta (spaghetti) quality characteristics, referring to a loss of gluten quality, cooking values and a deterioration of sensory properties, when semolina from bug-damaged wheat is used to make pasta (Ozderen *et al*., 2008; Köksel *et al*., 2009). Similar consequences could be expected when baking certain traditional breads with flour obtained from damaged durum wheat, although no test has yet been performed to assess this hypothesis.

The final conclusion is that wheat bugs have a strong influence on durum wheat proteins that determines a quality loss. When hydrating durum wheat flour or semolina obtained from kernels damaged by wheat bugs the glutenins depolymerised quickly and almost in their totality. This degradation is independent of temperature. By contrast, the gliadins and the solubilised glutenins are degraded with less intensity and the degradation is time and temperature dependent.

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