

Effects of Wheat Bug (*Eurygaster maura*) Protease on Glutenin Proteins

D. Sivri,¹ H. D. Sapirstein,^{2,3} H. Köksel,¹ and W. Bushuk³

ABSTRACT

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Proteolytic degradation of 50% 1-propanol insoluble (50PI) glutenin of six common wheat cultivars by wheat bug (*Eurygaster maura*) protease was investigated using reversed-phase HPLC. Wheat at the milk-ripe stage was manually infested with adult bugs. After harvest, bug-damaged kernels were blended (2:1, kernel basis) with undamaged grain of the same cultivar. Samples of ground wheat were incubated in distilled water for different times (0, 30, 60, and 120 min). The incubated whole meal samples were subsequently freeze-dried and stored until analysis. The degree of proteolytic degradation of 50PI glutenin was determined based on the quantity of total glutenin subunits (GS), high molecular weight GS (HMW-GS), and low molecular weight GS (LMW-GS). For ground wheat samples incubated for ≥ 30 min, 50PI glutenin was substantially degraded as evidenced

by a >80% decrease on average in total GS, HMW-GS, and LMW-GS. Some cultivars showed different patterns of glutenin proteolysis as revealed by differences in the ratios of HMW-GS to LMW-GS between sound and bug-damaged samples; a significant decrease in this ratio was found for four cultivars. This evidence, combined with other observations, indicated that there were intercultivar differences in polymeric glutenin resistance to the protease of the wheat bug *Eurygaster maura*. While the nature of this resistance is unknown, it should be possible to select and develop wheat cultivars with improved tolerance for wheat bug damage. Propanol insoluble glutenin, which corresponds to relatively large glutenin polymers, appears to be an excellent quantitative marker for this purpose.

Preharvest bug damage to wheat caused by *Eurygaster* spp. and *Aelia* spp. that results in substantial losses in yield and quality occurs in most countries of the Middle East, Eastern Europe, and North Africa (Paulian and Popov 1980). Wheat grain infested by these bugs contains a protease that breaks down the structure of gluten, causing a softening of the dough and subsequently giving bread of low volume and unsatisfactory texture (Kretovich 1944). In New Zealand, similar wheat bug damage has been associated with another insect, *Nysius huttoni* (Every et al 1998); proteases of this insect appear to specifically hydrolyze the high molecular weight glutenin subunits (HMW-GS) (Cressey et al 1987, Swallow and Cressey 1987). The hydrolyzing effect of the bug enzyme on gluten proteins was determined by acid (A) PAGE and SDS-PAGE (Kozmina and Tvorogova 1973, Yakovenko et al 1973, Cressey and McStay 1987, Sivri and Köksel 1996). There have been some attempts to quantitate the activity of *N. huttoni* proteolytic enzymes (Every 1991). However, we are not aware of any reports of similar studies on *Eurygaster maura* protease.

We recently reported (Sivri et al 1998) on the effects of *E. maura* protease on wheat proteins. In that study, the effects of bug damage on six Turkish wheat cultivars were subjectively evaluated based on A-PAGE of gliadins and SDS-PAGE of reduced insoluble glutenin; both protein fractions, depending on cultivar and sample incubation time, showed varying and substantial effects of bug damage. The main aim of this study was to quantitatively determine, by reversed-phase (RP) HPLC, the effects of bug damage on the high molecular weight (HMW) and low molecular weight (LMW) subunit composition of 50% 1-propanol insoluble (50PI) glutenin. Insoluble glutenin is widely considered the most important protein fraction of wheat related to intercultivar differences in breadmaking quality (see review by Weegels et al 1996).

MATERIALS AND METHODS

Wheat Cultivars and Bug Damage

Six common wheat cultivars of diverse breadmaking quality were selected from the 1994 crop grown on experimental plots near Ankara, Turkey. These included three hard red winter wheat cultivars (Bezo-

staya, Lancer and Gun), one medium-hard white winter wheat (Kirkpınar), and two soft white winter wheats (Ankara and Kirac). When the wheats were at the milk-ripe stage, a 1-m² portion of the plot was covered by nylon gauze cages and manually infested with 500 field-collected adult *E. maura* bugs. The infestation was allowed to persist until harvest. Wheat kernels showing characteristic puncture marks (black spots surrounded by pale, slightly sunken patches) were selected by hand-picking. The protein (N \times 5.7, 14% mb) contents of the wheat samples, determined by the Kjeldahl method (AACC 1995) were in the range 13.3–16.3%; there was no more than 0.6% difference in protein content between sound and bug-damaged wheat of the same cultivar.

Bug-damaged kernels were blended with undamaged grain of the same cultivar (2:1, kernel basis) and were ground in a coffee grinder to obtain whole meal passing through a 100-mesh sieve. Ground wheat (40 mg) was incubated with 200 μ L of distilled water for 0, 30, 60, and 120 min at 37°C in a water bath. After incubation, the whole meal samples were immediately frozen and freeze-dried, and stored at 4°C until HPLC analysis.

RP-HPLC Analysis

Samples were prepared according to Fu and Sapirstein (1996). Freeze-dried unincubated or incubated ground wheat (40 mg) were extracted twice with 1 mL of 50% (v/v) 1-propanol for 30 min at 23°C and centrifuged for 3 min at 2,200 \times g. The residues were washed with the same solution for 1 min and centrifuged for 3 min at 15,000 \times g; the supernatants were discarded. The precipitates were reduced with 0.1 mL of freshly prepared solution of 1% (w/v) dithiothreitol (DTT) in 0.08M Tris-HCl buffer (pH 7.5) containing 50% (v/v) 1-propanol for 60 min at 60°C. The reduced glutenin was alkylated with 0.1 mL of 14% (v/v) 4-vinylpyridine prepared in 0.08M Tris-HCl (pH 7.5) containing 50% (v/v) 1-propanol. Alkylation was carried out at 60°C for 15 min, and was followed by centrifugation for 5 min at 15,000 \times g. The supernatants were syringe-filtered (0.45 μ m Millex HV) into HPLC microvials.

RP-HPLC of reduced 50PI glutenin was conducted (Fu and Sapirstein 1996) using a Hewlett Packard 1090 M liquid chromatograph and a Zorbax 300 SB-C8 (300 Å pore size, 5- μ m particle size) analytical column (4.6 \times 150 mm) and a guard column of similar composition. Solvents for column elution were deionized, filtered, and deaerated distilled water and acetonitrile. Both solvents contained 0.1% (v/v) trifluoroacetic acid. Elution was at a flow rate of 1 mL/min using gradient conditions as previously specified (Fu and Sapirstein 1996). Absorbance of eluted proteins was monitored at 214 nm. Data quantitation was performed using chromatograph data analysis software (Hewlett Packard HPLC Chemstation ver. A.05).

¹ Hacettepe University, Faculty of Engineering, Food Engineering Department, 06532 Beytepe, Ankara, Turkey.

² Corresponding author. E-mail: harry_sapirstein@umanitoba.ca

³ The University of Manitoba, Department of Food Science, Winnipeg, MB, Canada, R3T2N2.

Chromatogram integration parameters were uniformly applied to all chromatograms to quantitate total GS, HMW-GS, and LMW-GS. As previously reported (Fu and Sapirstein 1996), HMW-GS and LMW-GS eluted at 24–46 min and 52–82 min, respectively. Total GS was determined as the sum of integrated areas of HMW-GS and LMW-GS. Representative examples of chromatograms of sound and bug-damaged samples (0 and 30 min of incubation) are shown in Fig. 1.

The relative effect of bug damage among cultivar samples based on the RP-HPLC analysis was calculated as $(100 - 100 \times D_c/S_c)$, where D_c is the integration value for total GS, HMW-GS, or LMW-GS for damaged wheat of a given cultivar at different incubation periods, and S_c is the corresponding value for the sound wheat sample of the same cultivar.

Statistical Analysis

All results reported here are the means of at least two replicates. Results were analyzed by the one way analysis of variance procedure using the MSTAT-C statistical software (Michigan State University, Lansing, MI) or SAS/STAT System software ver. 6.12 (SAS Institute, Cary, NC). For comparison among means, significant differences were calculated at $P < 0.05$.

RESULTS

The effects of bug damage on the reduced 50PI glutenin fraction as determined by RP-HPLC are presented in Fig. 2. Compared with sound wheat, for bug-damaged samples incubated for 0 min, there was relatively little or no reduction in the amounts of total GS, HMW-GS, and LMW-GS. For some cultivars (Bezostaya, Lancer, and Kirkpinar), these differences were statistically significant. The preparation of the bug-damaged samples with the 0-min incubation treatment involves the addition of water to the ground wheat, followed by vortexing and immediate freezing and freeze-drying. Although the initial freezing is intended to stop the proteolytic activity, the enzyme might be active for a short period affecting the gluten structure. An example of the effect of minimal incubation on 50PI glutenin subunits resolved by RP-HPLC is shown in Fig. 1B for cultivar Lancer. Compared with the corresponding sound wheat (Fig. 1A), a noticeable decrease in the overall quantity of HMW-GS and LMW-GS was observed. This decrease can be attributed to the potency of the proteolytic enzyme from *E. mauro* or the susceptibility of large glutenin polymers, such as 50PI glutenin, to its effects.

The ability of the bug protease to degrade 50PI glutenin of all cultivars was considerable, as shown by the substantial decrease in total GS, HMW-GS, and LMW-GS at 30-min incubation time (Fig. 1C, Fig. 2). For total GS, this decrease at 30-min incubation compared with sound wheat was $\approx 82\%$ on average (Table I). With further incubation time (>30 min), a slight decreasing trend in total GS, HMW-GS, and LMW-GS was observed (Fig. 2). However, in general, this decrease was not significant ($P < 0.05$) as most of the degradation of glutenin occurred by 30 min. Clearly, distilled water was an effective medium to incubate ground samples of bug-damaged wheat. Distilled water, rather than a buffer solution, was chosen for these experiments for a number of reasons. While preliminary results (Sivri 1998) indicated that bug protease had an optimum activity at pH 8.5 in 0.05M Tris-HCl buffer using vital wheat gluten as the substrate, the activity was stable over a wide pH range (pH 3-10). We also found that different buffer solutions affected the protease activity differently. As the practical effects of bug damage on wheat relate first to the technological properties of flour-water doughs, using water as an incubation medium to evaluate bug-damage effects on glutenin was a convenient basis to obtain results that could be interpreted most easily.

Accompanying the decreases in total GS, HMW-GS, and LMW-GS, variable results were found regarding the composition of glutenin in terms of the ratio of HMW-GS to LMW-GS. Whereas no consistent effect of bug damage was observed for Bezostaya,

three of the cultivar samples (Ankara, Kirac, and Gun) had a small, but significant decrease in this ratio between sound wheat and bug-damaged samples incubated ≥ 30 min. For Lancer, the corresponding decrease in the ratio of HMW-GS to LMW-GS was much larger ($\approx 50\%$). As the molecular weight distribution of glutenin is believed to shift toward higher molecular weights as the ratio of HMW-GS to LMW-GS increases (MacRitchie 1992), this decrease in the ratio of HMW-GS to LMW-GS suggests that bug damage had a greater effect on the largest polymers of glutenin. A similar effect on the ratio of HMW-GS to LMW-GS in 50PI glutenin was observed in gamma-irradiated wheat (Köksel et al 1998). Interestingly, for Kirkpinar, bug damage at longer incubation times resulted in a significant and progressive increase in the ratio of HMW-GS

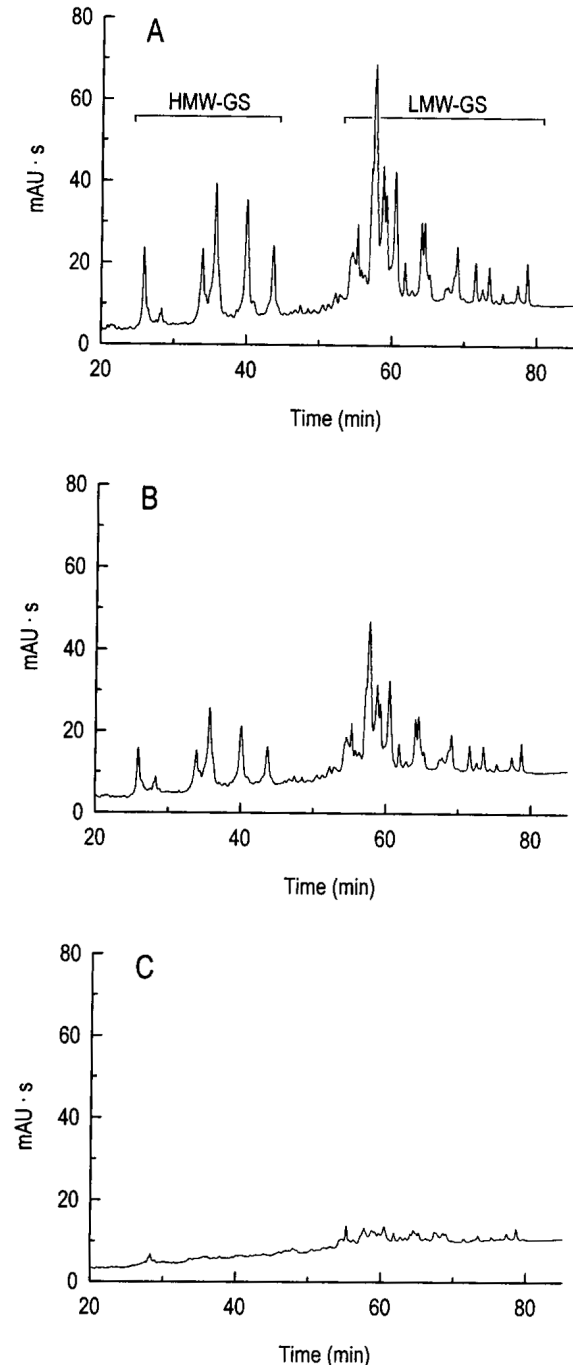


Fig. 1. Typical reversed-phase HPLC results of reduced 50% propanol insoluble glutenin for samples of sound (A) and bug-damaged wheat incubated for 0 min (B) and for 30 min (C).

to LMW-GS in 50PI glutenin. Why Kirkpinar was distinct in this way is unknown, but it suggests a glutenin structure or size distribution that is different from that possessed by the other cultivar samples in this study.

DISCUSSION

It has been previously reported (Sivri and Köksel 1996) that bug damage from *E. maura* had a marked effect on SDS-PAGE patterns of reduced glutenin in total protein extracts of the same wheat cultivars. In that study, the relative intensities of bands of glutenin subunits, especially those of the HMW-GS, decreased substantially as the incubation time increased. For the LMW-GS, it was more difficult to gauge the bug damage effects due to the comigration in SDS-

PAGE of gliadin bands in the total protein extracts. More recently (Sivri et al 1998), effects of *E. maura* damage on gliadin and glutenin proteins were separately examined by A-PAGE of gliadin and SDS-PAGE of reduced extracts of 50PI glutenin. The latter protein fraction was prepared, as in this study, to exclude all monomeric proteins, including gliadins (Fu and Sapirstein 1996). Progressive decreases in the staining intensities of gliadin and glutenin subunit bands were observed in the bug-damaged wheat as incubation time increased.

In this study, by using 50% 1-propanol to preextract the monomeric proteins along with soluble glutenin, and subsequently analyzing the reduced residues by RP-HPLC, direct quantitative measures of the degrading effects of *E. maura* bug damage were obtained. That the bug damage was proteolytic in nature appears to be clear. One of the authors (Sivri 1998) examined specific protease

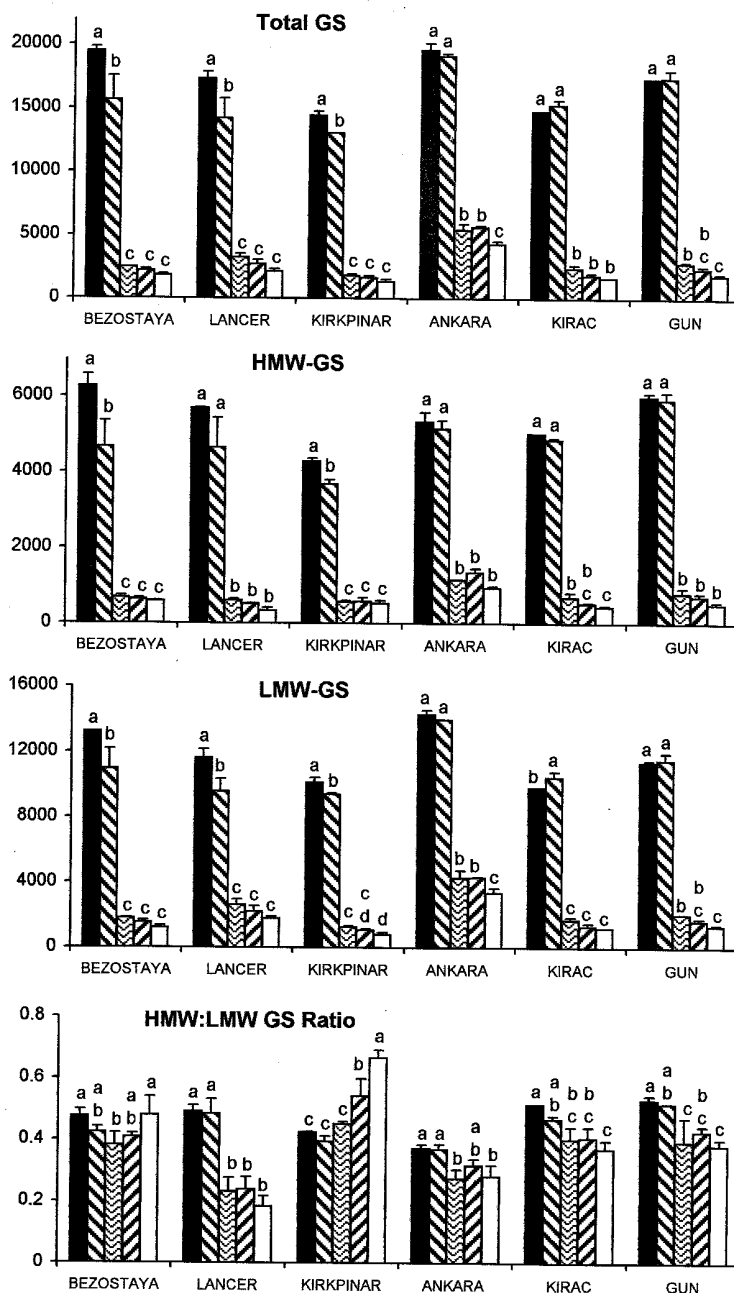


Fig. 2. Effect of bug damage on amount (in mAU · sec) of total glutenin subunits (GS), high molecular weight GS (HMW-GS), low molecular weight GS (LMW-GS), and the ratio of HMW-GS to LMW-GS determined by reversed-phase HPLC analysis of reduced 50PI glutenin isolated from six cultivar samples. For each cultivar and glutenin parameter, the data are grouped according to treatment from left to right: sound wheat; damaged wheat incubated for 0, 30, 60, and 120 min. Within each grouping, the same letter above a histogram bar indicates a treatment that is not significantly different ($P < 0.05$).

inhibitors such as soybean trypsin inhibitor (0.01M) and *p*-chloro-mercurybenzoic acid (PCMB) (0.01M) among others, on extracts of a sample heavily damaged by *E. maura*. Inhibition of protease activity was assayed using insoluble glutenin as a substrate (Sapirstein and Johnson 1996) similar to the approach adopted by Sivri et al (1998) to assay protease activity in bug-damaged wheats. We found that 67 and 100% of enzyme activity was inhibited by trypsin inhibitor and PCMB, respectively. Details of this work will be reported in a later article.

Scanning electron microscopy (SEM) examination of bug-damaged and sound kernels of the six wheat cultivars revealed considerable differences in endosperm structure as a result of degrading effects of bug protease (Sivri 1998). In the damaged area, the protein matrix was completely destroyed and starch granules could be clearly seen. In contrast, the protein matrix in the undamaged sections of the kernel appeared normal. These results indicate that the protein matrix is degraded by the *E. maura* protease activity. The SEM views of bug-damaged kernel were similar to those reported by Nightingale et al (1999) for bread and durum wheats infected by *Fusarium* spp. Our results on the effects of *E. maura* protease on polymeric glutenin are also analogous to those obtained by Nightingale et al (1999), who found *Fusarium* protease caused a large decrease in HMW protein (presumably glutenin) eluting in the void fraction of size-exclusion chromatograms of total wheat storage proteins of incubated treatments.

Our results are also similar to those obtained in New Zealand wheats damaged by the wheat bug *N. huttoni*, which is known to have detrimental effects on dough rheological properties and baking quality (Cressey and McStay 1987). They reported that hydrolysis of gluten proteins was endo-proteolytic, and the enzyme had a higher specificity for the HMW-GS than for the other gluten proteins. The decrease in the ratio of HMW-GS to LMW-GS, as observed in the present study, for four of six cultivar samples partially confirms this earlier observation.

Although we found significant decreases in total GS, HMW-GS, and LMW-GS contents caused by bug protease, it was not possible to directly compare these data for intercultural differences because the initial levels of 50PI glutenin were significantly different among these cultivars (Table I). For example, one cultivar (Ankara) had a significantly higher quantity of 50PI glutenin and two cultivars (Kirkpinar and Kirac) had significantly lower amounts when compared with other samples. Accordingly, relative reductions in total 50 PI glutenin, HMW-GS, and LMW-GS concentrations were calculated to compare these cultivars for resistance to the bug protease (Table I). For all three glutenin parameters (total GS, HMW-GS, and LMW-GS), over all incubation times, Ankara was most distinct among all the cultivars tested in terms of having significantly lower levels of glutenin degradation.

It seems clear then that Ankara glutenin has been affected differently by bug protease. The nature of this difference might be

due to Ankara having a higher resistance to the proteolytic effects *E. maura*. Another possibility is a lower level of proteolytic activity in the bug-damaged wheat. This latter possibility was examined (Sivri et al 1998) by blending a small portion of bug-damaged wheat with 10× the amount of sound wheat and subsequently quantifying the proteolytic effect on large polymeric glutenin (50PI glutenin). The results indicated that the cultivars Bezostaya, Lancer, Ankara, and Gun were not significantly different in terms of overall proteolytic effects, which were relatively lower when compared with cultivars Kirac and Kirkpinar. Thus, the significantly higher resistance of Lancer glutenin to bug damage, or conversely, the relatively higher susceptibility of Bezostaya glutenin, cannot be readily explained by differences in proteolytic activity. Other possible genotypic factors such as kernel characteristics and wheat plant morphology were not investigated in this study. However, those factors were examined previously. Every et al (1996, 1998) showed that large differences existed in the resistance of various New Zealand genotypes to the effects of *N. huttoni* bug protease that could not be related to differences in wheat end-use (biscuit or bread quality), grain characteristics (color, hardness, and texture), and head characteristics (shape, awns, and waxiness). It seems plausible therefore, that the cultivar differences to the effects of bug protease found in this study might be ascribed to intrinsic differences in glutenin structure.

CONCLUSIONS

The detrimental effects of wheat bug (*E. maura*) protease, and intercultural differences, can be effectively determined using relatively large polymeric glutenin as a substrate (i.e., glutenin insoluble in 50% 1-propanol). We show quantitatively, for the first time using RP-HPLC, that bug damage can cause substantial decreases (>80% on average) in the amount of 50 PI glutenin and constituent subunits on incubation of ground wheat for 30 min. Both HMW-GS and LMW-GS are degraded by bug protease, and the extent of hydrolyzing effects appeared to be cultivar-specific. Some cultivars showed very different patterns of degradation as evidenced by differences in the ratio of HMW-GS to LMW-GS between sound and bug-damaged samples. It seems likely that intercultural differences exist in polymeric glutenin resistance to the protease of the wheat bug *E. maura*. While the nature of this resistance is unknown, it should be possible to select or develop wheat cultivars with improved tolerance for wheat bug infestation using 50% propanol insoluble glutenin as the quantitative marker.

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TABLE I
Effect of Bug Enzyme on the Relative Degradation of Total Glutenin Subunits (GS), High Molecular Weight GS (HMW-GS), and Low Molecular Weight GS (LMW-GS) Determined by Reversed-Phase HPLC^a

Cultivar	50PI Glutenin ^b (mAU·sec)	Total GS Incubation Time (min)			HMW-GS Incubation Time (min)			LMW-GS Incubation Time (min)		
		30	60	120	30	60	120	30	60	120
Bezostaya	22,681ab	87.3a	88.8a	90.7a	89.1a	90.0ab	90.7b	86.4ab	88.3ab	90.7ab
Lancer	21,585b	81.3b	84.2b	87.8b	89.4a	90.9a	94.2a	77.4c	81.0c	84.7c
Gun	21,168b	83.7ab	86.3ab	89.3ab	86.7a	88.1ab	91.4b	82.1b	85.3b	88.1b
Kirkpinar	17,366c	87.4a	88.5a	90.7a	86.8a	86.4b	87.5c	87.6a	89.4a	92.0a
Ankara	23,541a	72.4c	71.3c	78.1c	78.3b	74.7c	82.4d	70.1d	70.0d	76.5d
Kirac	18,344c	83.7ab	87.5a	88.9ab	86.2a	89.5ab	91.2b	82.4b	86.5ab	87.7a

^a Total GS, HMW-GS, and LMW-GS values are expressed as percentage decreases. Means within columns followed by the same letter are not significantly different ($P < 0.05$).

^b 50% 1-propanol-insoluble (50PI) glutenin (total GS) in sound wheat.

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