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The Effect of Bug Damage on Physicochemical, Electrophoretic and Quality Factors of Wheat Gluten

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ABSTRACT: One of the most important forms of preharvest damage to wheat is caused by sunn pests. The insects insert their mouth parts into the immature grain and while injecting their saliva suck the milky juices. Flour from damaged wheat results in low baking performance due to the bug proteolytic enzymes' injected which cause the breakdown of gluten structure in the dough. In the present study three wheat varieties named Alvand, Mahdavi and Roshan with different levels of bug damage were studied. The effect of bug damage on physicochmical properties showed reduced thousand kernel weight, protein and ash contents. Gluten and gluten index decreased significantly upon bug damage. Among the factors the degree of gluten hydrolysis, total reducing substances and sulfhydryl groups content were increased upon bug damage in all the three varieties examined but disulfide groups content was reduced. Monitoring of the wheat flour protein by SDS-PAGE indicated that some of the bands related to HMW-GS at the top of electropherogram were disappeared and the intensity of some other were decreased. In Roshan and Alvand varieties, the intensity of bands with similar mobility to gliadins was decreased. Incubation of bug-damaged samples resulted in further degradation of electropherogram bands of high molecular weights and development of bands with lower molecular weights that is due to the hydrolyzing effect of bug protease.

Keywords: *Gluten, SDS-PAGE, Sunn Pests, Sulfhydryl Groups.*

Introduction

Damage to bread-making quality of wheat, due to preharvest heteroproteus insects' attack, has been widespread in many countries of the Middle East, Eastern Europe and North Africa (Bonet *et al.*, 2005; Karababa and Ozan, 1998). The insects, called Sunn pests, or wheat bugs (*Eurygaster* spp. and *Aelia* spp.), insert their mouth parts into the immature grain and while injecting their saliva suck the milky juices. The visible effect of this attack is dark spots (points of stylet penetration) with a surrounding pale area in which the protein matrix is absent (Aja *et al.*, 2004; Every *et al.*, 1998). Flour from damaged wheat leads to sticky and weak doughs formation and loaves with low volume and unacceptable texture (Cressey and McStay, 1987). Deterioration of the quality is due to proteolytic enzymes injected into the kernels by insects via their saliva that persist in the flour after milling and cause breakdown of gluten structure in the dough. The detrimental effect on baking quality is obvious in the presence of 3-5٪ damaged kernels and dramatically is increasing to the amounts higher than 10٪ (Vaccino *et al.*, 2006). The insect infestation affects the glutenin and gliadin fractions of wheat protein, with increased specifity towards the high molecular weight glutenin subunits (Every *et al.*, 1998 b; Cressey *et al.*, 1987; Perez *et al.*, 2005), therefore the unique

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characteristic of bug-damaged wheat, is a disrupted protein structure (Aja *et al.,* 2004).

In the present study, the effect of bug damage on physicochemical properties of wheat flour and gluten quality is investigated and indeed the effect of bug enzyme on the flour protein is shown by SDS-PAGE.

Materials and Methods

- *Wheat samples*

Three Bug-damaged wheat varieties, Alvand (white, strong), Mahdavi (white, intermediate quality) and Roshan (white, intermediate to weak quality), were obtained from the farming fields of the university that had been cultivated in cages and been exposed to Eurygaster integriceps feeding. Harvested wheat kernels, were the blend of sound and damaged kernels in different three varieties with different levels of damaged kernels. Sound and damaged kernels were separated from these samples manually and used for the experiments. Damaged kernels had obvious dark spot and a pale area around the insect's sting.

- *Flour preparation*

Wheat kernels were grounded into whole meal by laboratory mill.

- *Bug damage percent*

The percentage of bug damage was determined by manually selecting and weighing the infested kernels with the black spot in a 50g sample of wheat kernels consignment for each variety.

- *Thousand kernel weight*

Thousand kernel weights were determined by weighing one thousand grains randomly separated from the wheat samples (Mariotti *et al.,* 2006). Moisture, ash and protein contents were measured according to AACC 44-16, 08-01 and 46-12 standard methods (2003) respectively.

- *Reagents*

All reagents used, were from Merck Company, except o-phthaldialdehyde which was purchased from Sigma.

- *Wet and dry gluten and gluten index*

Wet gluten was measured according to AACC 38-10 standard procedure (2003) using hand-washing method and then was dried for determinnig dry gluten. Gluten index was determined according to 38-12A standard method of AACC (2003).

- *Total reducing substances*

Total reducing substances (TRS) was determined according to AACC 10-01 standard method (1995) on bug-damaged and sound types of wheat varieties. As glutathione and cysteine content falls during storage of flour (Grosch and wiesser, 1999), TRS was determined in freshly-ground wheat. The reducing substances was extracted from 2g of flour with 25 ml 10% TCA for 15 min at room temperature, stirring occasionally, centrifuged at 362×g for 10 min. Aliquot of supernatant liquid added to 3 ml 0.005 N iodine solution. After 5 min, excess iodine was titrated with 0.005 N thiosulfate. The following equation was used for calculation of TRS:

TRS= [3- (ml thiosulfate consumed)] (1)

- *Degree of gluten hydrolysis*

Bug-damaged degree of gluten hydrolysis (DGH) was measured using o-phthaldialdehyde (OPA) method, which determines changes in free amino groups. In this method, reaction between free amino groups and o-phthaldialdehyde (OPA) in the presence of beta-mercaptoethanol forms a colored compound detectable at 340 nm in a spectrophotometer (Nielsen *et al* 2001). Briefly cold-air-dried gluten (25mg) was suspended in 1 ml of 0.1 M KCl solution with pH of 1.0 then vortexed for 5 min and centrifuged at 15700×g for 5 min. 50 µl of the clear supernatant was added to 250 µl of OPA reagent containing 0.25% (v/v) 2-mercaptoethanol and after 2 min the

absorbance was read at 340 nm in a single lamp UV spectrophotometer type 2100 Unico. Results of free amino groups (µg of serine) in g of gluten were calculated against a serine standard curve (Bonet *et al.*, 2005), and then DH was calculated using the following equations:

$$DH = (h/h_{tot}) \times 100 \quad (2)$$

$$h = [(\text{Serine-}NH_2) - \beta] / \alpha$$

$$h_{tot} = 8.3$$

where DH is the degree of hydrolysis, h_{tot} is the total number of peptide bonds per protein equivalent, and h is the number of hydrolyzed bonds. Values of α, β and h_{tot} for different raw materials are constant and for gluten are respectively, 1.00, 0.40 and 8.3 (Nielsen *et al.*, 2001).

- *Sulfhydryl (SH) and disulfide (SS) groups quantification*

SH and SS content of freshly-grounded whole meal four, in both sound and bug-damaged samples were determined according to Beveridge *et al.* (1974) using Ellman's reagent, which reacts with thiol group and forms 2-nitro-5-thiobenzoic acid dianione that absorbs 412 nm (Riddles *et al.*, 1979).

In this method 75 mg (on dry basis) of flour was dispersed in 1 ml of Tris-glycine buffer (pH 8). 4.7g Guanidine hydrochloride was added and the volume made to 10 ml. For SH, to 1ml of this slightly turbid solution was added 4ml of 8M urea containing 5M Guanidin hydrochloride in Tris-glycine buffer solution (Urea-GuHCl) and then 50 µl of Ellman's reagent was added. For SS, to 1ml of the reaction mixture was added 0.05 ml 2-mercaptoethanol and 4 ml of Urea-GuHCl and the mixture was incubated for 1 hour at 25ºC. After an additional 1 hour incubation with 10ml of 12% TCA, the tubes were centrifuged at 5000×g for 10 min. The precipitate was twice resuspended in 5ml of 12% TCA and centrifuged to remove 2-mercapoethanol. The precipitate was dissolved in 10ml of 8M urea in Tris-Glycine, and the color was developed with 40 µl of Ellman's reagent. SH and SS contents were calculated as follows:

$$\mu M\ SH/g = \frac{73.53 \times A_{412} \times D}{C} \quad (3)$$

A_{412}: Absorbance at 412 nm
C: Sample concentration in mg solids/ml
D: Dilution factor, 5.02 for SH and 10 for total SH (SH + reduced SS)
SS content is µM SH content after reduction minus SH content before reduction.

- *Electrophoresis*

SDS-PAGE experiment was carried out on the samples according to Every *et al.* (1998) method.

- *Statistical analysis*

The results were analyzed using the SPSS package (version 14). Analysis of variance was done with ANOVA. Least significant differences (LSD) test was used to describe means at the 5% significance level.

Results and Discussion

- *Thousand kernel weight*

Damaged kernels had significantly lower thousand kernel weight than sound ones ($p<0.05$) (Table 1). During the early stages of kernel development (e.g., milk-ripe stage), much of the kernel content might be sucked out by the insect, resulting in small, light and shriveled kernels. The resulting mature kernels are partially empty, especially with respect to the protein matrix in the region of the grain around the puncture site (Rosell *et al.*, 2002). Thus the overall characteristics of bug-damaged wheat such as test weight and thousand kernel weights are reduced when the percentage of damaged kernels increases (Karababa and Ozan, 1998).

Table1. Physical and chemical characteristics of the wheat varieties [1]

Characteristics / Varieties		BDP[2, 3] (%)	TKW[2, 4] (g)	M[2, 5] (%)	A[2, 6] (%)	P[2, 7] (%)
Alvand	Sound	0	41.9^{a}±0.8	7.8^{a}±0.2	1.7^{a}±0.1	12.3^{a}±0.2
	Damaged	100	36.9^{b}±0. 7	7.7^{a}±0.1	1.7^{a}±0.1	11.8^{a}±0.2
	Blend	4.9±0.3	35.1^{c}±0.9	8.0^{b}±0.1	1.6^{a}±0.0	12.4^{a}±0.4
Mahdavi	Sound	0	36.7^{a}±0.2	6.2^{a}±0.1	1.6^{a}±0.0	12.5^{a}±0.4
	Damaged	100	35.5^{ab}±0.9	6.0^{a}±0.2	1.7^{b}±0.1	11.7^{b}±0.2
	Blend	11.9±0.8	35.0^{b}±0.5	6.1^{a}±0.3	1.7^{a}±0.1	11.9^{a}±0.7
Roshan	Sound	0	41.6^{a}±0.4	6.1^{a}±0.1	1.7^{a}±0.1	11.4^{a}±0.3
	Damaged	100	37.8^{b}±0.9	6.0^{a}±0.1	2.0^{b}±0.1	10.3^{b}±0.3
	Blend	9.2±0.6	33.4^{c}±0.3	6.0^{a}±0.1	1.9^{c}±0.1	10.9^{b}±0.5

1: Different small letters in each column for each variety are significantly different at $p<0.05$.
2: BDP, TKW, M, A and P are abbreviations of Bug Damage Percent, Thousand Kernel Weight, Moisture, Ash and Protein, respectively.
3, 4, 5, 6, and 7: Values are means of three replicates ± standard deviation.

- *Moisture, Ash and Protein contents*

Moisture content of sound, damaged and blend types of varieties (Table 1) were not significantely ($p<0.05$) different as expected because they were kept together before experiments.

Ash and protein contents are significantely ($p<0.05$) affected by bug damage in varieties with high bug damage percent (Mahdavi and Roshan), but not in Alvand with lower bug damage percent. According to Najafi-Mrak (2012), sunn pest reduced grain protein content.

- *Degree of gluten hydrolysis*

Bug damage has significantly ($p<0.05$) increased the degree of gluten hydrolysis, as the DGH of bug-damaged kernels in Alvand, Mahdavi and Roshan was 2.6, 2.8 and 2.5 times greater than that of sound kernels of the same varieties, respectively (Table 2). It has been also reported a two-fold increase of DGH over sound gluten due to the hydrolysis activity of the insect proteases present in damaged wheat (Bonet *et al.*, 2005).

Table 2 shows gluten hydrolysis in the sound samples which is probably due to their natural proteases.

Perez *et al*. (2005) in an attempt to determine gluten degradation of bug-damaged kernels during incubation at 37ºC, showed that free amino groups are increased along with incubation time in a way that its concentration did not change during the first hour of incubation, but, after that, a steady increase was observed. The same trend was also evidenced for sound gluten although at a lower rate.

Table 2. Degree of gluten hydrolysis[1, 2, 3]

Sample	Alvand	Roshan	Mahdavi
Sound kernels	0.85$^{c\,B}$ ±0.05	1.24$^{c\,A}$ ±0.04	0.94$^{c\,B}$ ±0.09
Blend	1.49^{b}±0.09	2.50^{b}±0.10	1.89^{b}±0.10
Bug-damaged	2.25$^{a\,C}$ ±0.10	3.13$^{a\,A}$ ±0.09	2.61$^{a\,B}$ ±0.13

1: Values are means of three replicates ± standard deviation
2: Values followed by different small letter in each column are significantly different ($p<0.05$).
3: Values of different capital letter in each row are significantly different ($p<0.05$).

- *Wet and dry gluten*

Wet and dry gluten was decreased upon bug damage significantly ($p<0.05$) in all the three varieties (Table 3). This reduction is attributed to the proteolytic activity of enzyme injected into wheat which has disrupted protein structure of the grain (Torbica *et al.*, 2007; Karababa *et al.*, 1998).

Although bug damage percentage was higher for Mahdavi variety, but the wet and dry gluten content were lower in Roshan due to its higher degree of hydrolysis. This shows that the percentage of damaged kernels might be not a suitable measurement for judgement about quality parameters among samples and the degree of hydrolysis should be initially determined for the comparison.

- *Gluten Index*

Bug damage significantly lowered gluten index (Table 3). This is due to gluten hydrolysis that allows the low molecular weight compounds to pass through the instrument sieve. Torbica *et al.* (2007) indicated a gluten index decrease upon bug damage.

- *Total reducing substances*

TRS values of bug-damaged flour in all the three varieties were obviously higher than the sound ones (Table 4). This could be due to the presence of proteolytic activity in bug-damaged flour. According to Aja *et al.* (2004), the compounds released in wheat upon bug-damage, could be peptones, peptides and amino acids. Eventually the proteolytic activity presents in the damaged wheat, increase low molecular weight protein contents which have more cysteine amino acids (Bonet *et al.*, 2005).

Table 3. Effect of bug damage on wet gluten, dry gluten and gluten index of wheat varieties[1, 2] (%)

Varieties	Characteristics	Sound	Blend	Bug-damaged
Alvand	Wet gluten	$31.7^{a}\pm1.4$	$21.3^{b}\pm1.8$	$7.4^{c}\pm0.4$
	Dry Gluten	$11.3^{a}\pm0.3$	$7.0^{b}\pm0.7$	$1.9^{c}\pm0.3$
	Gluten Index	$33.7^{a}\pm1.3$	$22.8^{b}\pm0.6$	$16.5^{c}\pm0.5$
Mahdavi	Wet gluten	$29.0^{a}\pm0.2$	$17.7^{b}\pm0.2$	$5.8^{c}\pm0.3$
	Dry Gluten	$10.0^{a}\pm0.1$	$5.5^{b}\pm0.1$	$1.9^{c}\pm0.2$
	Gluten Index	$28.9^{a}\pm0.5$	$18.6^{b}\pm0.9$	$11.0^{c}\pm0.1$
Roshan	Wet gluten	$26.0^{a}\pm0.3$	$14.6^{b}\pm0.1$	$4.5^{c}\pm0.4$
	Dry Gluten	$8.2^{a}\pm0.1$	$5.1^{b}\pm0.1$	$2.4^{c}\pm0.3$
	Gluten Index	$26.2^{a}\pm0.1$	$18.5^{b}\pm0.2$	$9.7^{c}\pm0.2$

1: Values are the mean of three replicates ± standard deviation.
2: Different letters in each row are significantly different at $p<0.05$.

Table 4. Effect of bug-damage on total reducing substances (ml) [1, 2, 3]

Samples	Alvand	Roshan	Mahdavi
Sound	$0.64^{e\,C}\pm0.03$	$0.87^{d\,A}\pm0.03$	$0.76^{e\,B}\pm0.02$
Blend	$1.29^{b}\pm0.04$	$1.52^{b}\pm0.05$	$1.49^{b}\pm0.04$
Bug-damaged	$1.47^{a\,C}\pm0.03$	$1.81^{a\,A}\pm0.04$	$1.67^{a\,B}\pm0.02$

1: Values are the mean of three replicates ± standard deviation.
2: Different small letters in each column are significantly different ($p<0.05$).
3: Different capital letters in each row are significantly different ($p<0.05$).

- *Sulfhydryl and disulfide groups*

In this study, SH content of Roshan, Mahdavi and Alvand varieties was increased upon bug-damage (Table 5). Similarly in Bonet *et al*. (2005) studies, the amount of thiol groups (SH content) in damaged gluten was also significantly higher than that of undamaged gluten. Perez *et al*. (2005) noted that although the bug proteases and not reductases are responsible to the wheat damage, but still this increase in thiol content can be observed. This phenomenon could be ascribed to the proteolytic activity present in the damaged gluten that could make accessible some hidden groups or increase the content of low molecular weight proteins that have more cysteine amino acids (Bonet *et al*., 2005).

Disulfide groups of bug-damaged samples of Roshan, Mahdavi and Alvand were lower than their counterpart sound samples (Table 5). Increase in the amount of peptides containing more cysteine amino acids resulted in more interchain SS to be broken (Ewart, 1985), as cysteine has an ability to reduce disulfide bonds (Tsen 1966) increasing the number of free thiol groups. Hydrolysis proceeds with the LMW-GS degradation affecting the second level of organization formed by the interchain disulfide bonds between HMW-GS and LMW-GS (Perez *et al.,* 2005). Lower one thousand kernel weights in damaged wheat samples might also be a sign of protein hydrolysis (Table 1).

- *SDS-PAGE*

Electropherograms of sound and bug-damaged whole meals of the wheat varieties before and after incubation are shown in Figure 1. Comparison of sound and bug-damaged wheat flour protein electropherograms of all three varieties revealed that some of the bands related to HMW-GS at the top of electropherogram were disappeared and the intensity of some other were decreased. In Roshan and Alvand varieties, intensity of bands with similar mobility to gliadins was decreased. Therefore bug protease affected both gliadin and glutenin subunits.

Every *et al*. (1990), Swallow and Every (1991), Cressey *et al*. (1987), Every *et al*. (1998), Vaccino *et al*. (1990) and Sivri *et al*. (1999), suggested specific hydrolyzing effects of bug protease on HMW-GS. Perez *et al.* (2005) studies indicated when analyzing the hydrolysis kinetics of bug proteases by HPCE for glutenins, it is shown that the HMW-GS, was not detected beyond 7 hour of incubation, where as the LMW-GS exhibited an additional degradation at lower rates. Torbica *et al*. (2007) showed that wheat-bug attack caused variation in electropherogram patterns of glutenins and gliadins concerning their number of bands, their intensities and corresponding molecular weights. Rosell *et al*. (2002) concluded that

Table 5. Effect of bug-damage on SH and SS contents (µmole/g) [1, 2, 3]

Samples	Mahdavi	Roshan	Alvand	Mahdavi	Roshan	Alvand
	SH			SS		
Sound	$1.96^{e.B}$±0.01	$2.48^{e.A}$±0.06	$1.46^{e.C}$±0.06	$8.55^{a.B}$±0.1	$7.71^{a.C}$±0.3	$9.16^{a.A}$±0.0
Blend	5.22^{b}±0.12	5.70^{b}±0.06	4.24^{b}±0.15	5.39^{b}±0.1	4.44^{d}±0.0	6.78^{d}±0.1
Bug damaged	$6.09^{a.A}$±0.16	$6.72^{a.A}$±0.12	$5.11^{a.B}$±0.28	$4.50^{e.B}$±0.2	$3.59^{e.C}$±0.1	$5.24^{e.A}$±0.1

1: Values are means of two replicates ± standard deviation.
2: Different small letters in each column are significantly different at $p<0.05$.
3: Different big letters in each row of SH or SS contents are significantly different at $p<0.05$.

bug proteases hydrolyze glutenins, without any specifity for HMW or LMW glutenin subunits, whereas Sivri *et al*. (1998) showed that *Eurygaster spp.* affected both gliadins and glutenins, with HMW-GS more severely degraded.Incubation did not affect electrophoregrms of sound samples greatly. In all three varieties, some bands with higher mobility were developed upon incubation of sound samples. Perez *et al.* (2005) reported that glutenins from sound gluten were not significantly ($p<0.05$) affected during incubation determined by HPCE. Aja *et al*. (2004) when analyzing water soluble extracts of sound gluten by SDS-PAGE, found only a band with 35 KDa when sound gluten was incubated for 0, 3 and 7 hours. However, after 24 h, numerous and intense protein bands were appeared, which correspond to the hydrolysis products resulting from the intrinsic proteolytic activity of the sound gluten.

Incubation of bug-damaged samples resulted in further degradation of electropherogram bands of high molecular weights and development of bands with lower molecular weights which can be related to ω-gliadins. Development of these low molecular weight bands, is due to the hydrolyzing effect of bug protease that has decreased intensity of high molecular weight bands and developed compounds with low molecular weights in electropherogram.

Aja *et al*. (2004) SDS-PAGE studies showed new bands of 27-42 KDa at 3 hour incubation of damaged gluten. Rosell *et al.* (2002) showed that in damaged wheat, both incubated and unincubated electropherograms revealed the presence of some new peaks with mobilities similar to ω-gliadins (Rosell *et al.*, 2002).

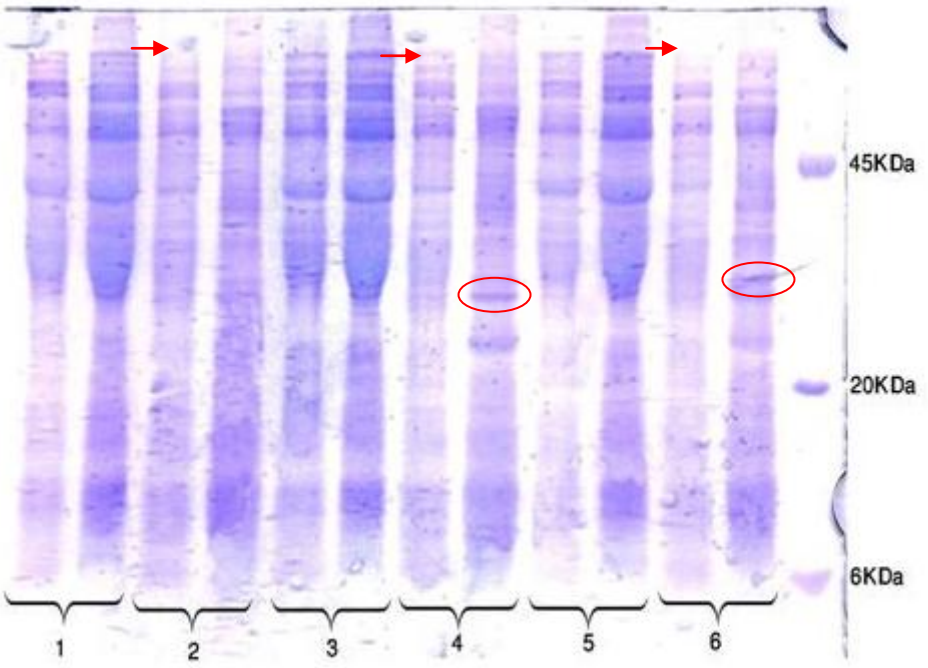

Fig.1. Electropherograms of bug-damaged and sound types of wheat varieties.
1: Sound Mahdavi, 2: bug-damaged Mahdavi, 3: sound Roshan, 4: bug-damaged Roshan, 5: sound Alvand, 6: bug-damaged Alvand
(In each number there are two lines which the line at the right side, is the sample in incubated condition)
Arrows indicate bands which are disappeared upon bug damage. Ovals indicate bands which are developed upon incubation of damaged kernels.

Conclusion

Bug protease degraded high molecular subunits of wheat gluten that is shown on electropherogram and produced free amino groups that were determined through DH analysis and subsequentely the internal SH groups will be accessible. Moreover and upon hydrolysis, the content of low molecular weight proteins such as glutathione with more cysteine amino acids will be increased. These SH groups have the ability to break SS bonds, therefore SH groups and total reducing substances are increased where as SS links are decreased. Such disrupted gluten structure have lower wet and dry gluten contents and gluten index than the sound samples.

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