REBUILDING GLUTEN NETWORK OF DAMAGED WHEAT BY MEAN OF GLUCOSE OXIDASE TREATMENT

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Running title : Glucose oxidase effect on insect damaged gluten

ABSTRACT

The disrupted gluten structure of infested wheat flours yields to low quality doughs unusable to bread-making processes. Enzymes are replacing chemical treatments in the food industry as a tool to treat weak flours. Glucose oxidase (GO) is one of the most promising oxidative enzymes although it has not been demonstrated yet its efficiency over the alcohol-soluble fraction of the gluten proteins. If this enzyme could restore the broken covalent bonds between the glutenin subunits, the gluten network of damaged wheat flour would recover their native structure and functionality. This treatment would allow bakers to use damaged flour, reducing the economical losses produced by this plague around Europe and North Africa. Electrophoretic studies demonstrated the formation of high molecular weight aggregates in the glutenin fraction, which had a characteristic thermal stability depending on the enzyme dosage. Those molecular studies agreed with the bread-making assays made with the maximum enzyme dosage and the microstructure determination. Overall results showed that GO is a real alternative to the traditionally used chemical oxidants. It acted specifically on the high molecular weight glutenin subunits of damaged wheat, forming dityrosine crosslinks between the wheat proteins, which reinforced the gluten network, and gave away the dough functionality.

Key words : glucose oxidase, damaged gluten, DSC, HPCE, texture parameters

INTRODUCTION

Preharvest infestation of wheat by Heteropterous insects produces a dramatic decrease in the gluten quality causing enormous losses to bakers, farmers and millers. The insect responsible of this plague in countries of Europe and North Africa are bugs of the genera *Eurygaster spp* and *Aelia spp*, and *Nysius huttoni* in New Zealand. Those insects inject salivary proteinases that solubilize the storage proteins, which are crucial for the formation of the gluten network and for its stability [1-3]. One of these proteinases was purified in wheat grains from New Zealand [4-6] but none of them has been purified yet in wheat grains from Europe or North Africa. The infestation of wheat grains produces the weakening of wheat proteins rendering softer dough that is unusable for industrial bread-making processes [7-9].

The baking industry standard treatments for weak flours include dough conditioners such as ascorbic acid, azodicarbonamide and potassium bromate [10]. The relationship between chemical oxidants and the incidence of cancer disease[11,12,13], as well as the recent importance that customers give to healthier products, are conducting companies to a progressive decrease in the use of chemical oxidants. Enzymes are the best and safest alternative to chemical compounds because they could be labelled as GRAS (generally recognized as safe), and do not remain active after baking because the high temperature of the process cause its denaturation and therefore the complete loss of activity. Enzymes are replacing chemical oxidants in numerous applications [14], having baking and animal feed industries the fastest growth rate in the use of enzymes over the past decade. The formation of covalent bonds between polypeptide chains (protein crosslinking) catalyzed by enzymes is a useful tool to reinforce the protein network [15].

Glucose oxidase (GO) [EC 1.1.3.4] is the most interesting oxidative enzyme in the food industry. It catalyzes the oxidation of β-D-glucose in the presence of O_2, producing D-

gluconic acid and a molecule of hydrogen peroxide that can either form disulfide bonds between proteins [16,17], or dityrosine crosslinks [18]. It has been already described the formation of dityrosine crosslinks by treating proteins with hydrogen peroxide or peroxidase [18-20]. Dunnewind et al (2002)[21] reported that the addition of GO increase the dough stress and the strain hardening. It also increases the loaf volume and improves the crumb grain of bread [22,23]. To summarize, it has been demonstrated that the addition of GO has a strengthening effect on wheat dough [24]. Diverse authors have detected an important effect of GO on the water-soluble protein fraction of flour, and it has been stated that the non-gluten proteins in dough systems are the most susceptible to oxidation [25]. Rasiah et al (2005)[26] reported that GO produced a slight improvement in crumb properties due to the crosslinking of the water soluble fraction. Vemulapalli and Hoseney (1998)[27] detected that the sulfhydril content of the water-soluble fraction extracted from flour and dough decreased in the presence of GO. Gujral and Rosell (2004)[28] demonstrated the ability of GO to modify also rice flour proteins, obtaining as a result better specific volume and texture of rice bread. Although the effect of GO on the water-soluble fraction of wheat proteins has been reported in several studies, the crosslinking of the glutenin fraction catalyzed by GO remains not completely understood.

Köksel et al (2001)[29] and Bonet et al (2005)[30] reported the improvement produced by transglutaminase (TG) on bug-damaged wheat flour by its rebuilding effect on the dough structure. Due to the important role that glutenins play in the gluten quality, the detection of crosslinking in the alcohol-soluble fraction would be particularly important to demonstrate the ability of GO to restore the structure and functionality of flour from insect damaged wheat. If GO could restore the disrupted gluten network of damaged wheat as TG did, it would be another tool available to reduce the economical losses caused to bakers and farmers.

The aim of this study was to restore the gluten functionality of infested wheat catalyzed by GO, to determine the possible thermal stabilization of the complex formed, and finally to demonstrate the importance of this effect on the improvement of the damaged flour bread-making properties. The protein thermal stabilization was followed by differential scanning calorimetry (DSC), the crosslinking between the glutenin subunits and the formation of high molecular weight aggregates was analyzed by high performance capillary electrophoresis (HPCE) and scanning electron microscopy (SEM), respectively. Changes produced in functionality of flour were assessed in bread loaves by mean of texture parameters, shape index (height/width) calculation, and specific volume of breads.

MATERIALS AND METHODS

Materials

Sound (9.1% protein) and insect damaged (9.9% protein) wheat flours were from cultivar Bolero. Wheat damaged was visually detected. The extent of the damage was quantified by using the Chopin Alveograph (Tripette et Renaud, Paris, France), as previously described Rosell et al (2002)[3]. One enzyme activity was arbitrarily defined as the reduction of deformation energy after 3 hr of incubation at 25°C, the damaged wheat showed 1.64mU/g of wheat (Aja et al 2004)[31].

Glucose oxidase (10000 glucose oxidase units [GU]/g) was kindly gifted by Novo Nordisk (Madrid, Spain). All reagents were of analytical grade.

Wheat samples after an appropriate cleaning were milled in a laboratory Perten Mill (Perten Instruments, Stockholm, Sweden).

Gluten samples preparation

Wet gluten balls were obtained from 10g of sound and damaged wheat flours following the AACC Approved Method 38-12a[32] by using a gluten washer (Glutomatic, Falling number, Stockholm, Sweden). Washed gluten was kept at 37ºC in a water bath for 30

min and then freeze-dried for further HPCE, DSC and SEM analysis. When GO was added, different GO concentrations (0.001, 0.005, 0.01g enzyme / 100g flour) were previously mixed with the flour.

Differential scanning calorimetry

The thermal behaviour of gluten proteins was determined as described in León et al (2003)[33] using a DSC (DSC-7 Perkin-Elmer, USA). Freeze-dried gluten samples (10mg) were weighted in aluminium pans (PE 0219-0062), and heated from 20 to 130ºC at 10ºC/min. An empty pan was used as a reference. Thermal transitions of gluten samples recorded were onset (To), peak (Tp) and conclusion (Tc) temperatures (ºC) and the denaturation temperature range (ΔTd =Tc-To). The enthalpy associated with protein denaturation (ΔHd) expressed as mJ/mg of sample was estimated by integrating the area under the endothermic peak. Four replicates were run for each sample.

High performance capillary electrophoresis analysis

A sequential extraction of the gluten proteins was performed following the Osborne method (1907), slightly modified by Bean et al (1998)[34] for isolating glutenins under reducing conditions, and gliadins. High molecular weight glutenin subunits (HMW-GS) and low molecular weight glutenin subunits (LMW-GS) were prepared from the glutenins by acetone precipitation as described Bean and Lookhart (2000)[35]. Four replicates of each sample were made.

Electrophoretic separations of the proteins were made using a Beckman MDQ instrument. Uncoated fused silica capillaries (Composite Metal Services Ltd, Worcester, UK) of 50μm i.d. x 27 cm (20 cm L/D) were used for all separations. High performance capillary electrophoresis was performed with 50mM iminodiacetic acid

(IDA) in acetonitrile and hydroxypropylmethylcellulose and water (20:0.05:79.95, v/v) at 45ºC and 30kV [35].

Cryo-scanning electron microscopy

SEM was used to examine the gluten structure. The freeze-dried gluten samples were mounted directly on stubs and coated with gold with a 35mA current in a sputter coater for 1 min. Then, were observed with a JEOL JSM-6300 scanning electron microscope with an accelerating voltage of 10kV.

Breadmaking and bread characteristics

Wheat flour (300g), salt (2%, flour basis), compressed yeast (3%, flour basis) and water (64%, flour basis) were blended in the bowl of the farinograph (Brabender, Germany). GO (when added) was incorporated at level of 0.01% (flour weight basis) to the flour before mixing. Dough was optimally mixed for 7 min, divided into 50g pieces, put in well-greased pans, proofed for 2 hour at 29ºC and 80% relative humidity and baked in an electric oven at 170ºC for 20 min. Bread was removed from the pans and cooled at 25°C for 2 hours.

Bread quality evaluation was carried out by measuring weight, volume (determined by seed displacement), specific volume, height/width ratio of the central slices. Crumb hardness was measured in a Texture Analyzer TA-XT2i (Stable Micro Systems, Surrey, UK) after 2 hours of cooling. A bread slice of 2 cm thickness was compressed to 50% of its original height at a crosshead of 1 mm/s with a cylindrical ebonita probe having a diameter of 10 mm. The peak force of compression was reported as hardness. Means are obtained from six replicates.

Statistical analysis

Multiple sample comparison was statistically analyzed with Statgraphics Plus 5.0. Duncan's multiple range tests was used to separate means. Significance was accepted at $P \leq 0.05$.

RESULTS AND DISCUSSION

Glucose oxidase effect on gluten from damaged wheat

The treatment of damaged gluten with the different GO levels on fractions was followed by HPCE (Figure 1). Total glutenins showed a significant ($P < 0.05$) decrease from non-treated samples to those treated with 0.001 and 0.005% GO. Nevertheless the most important effect on these subunits was detected with the highest GO dosage which value decreased more than four times in comparison with data obtained for non-treated gluten.

Gliadins significantly ($P < 0.05$) increased with the addition of GO, although increasing GO levels did not result in further enhancement. Michon et al (1999)[36] reported the ability of gliadins to form dityrosine crosslinks catalyzed by peroxidases. The gliadin aggregates formed as a result of the oxygen peroxide produced by the GO might not have exactly the same “space-filling” role as the gliadin monomers, due to its higher molecular weight. GO treatment may exclude part of the gliadins from the gluten network facilitating its extractability during the fractionation process, and therefore its detection by HPCE.

LMW-GS showed a significant ($P<0.05$) decrease when GO concentrations higher than 0.001% were added. HMW-GS had different tendency, showing a significant ($P<0.05$) decrease even in the presence of 0.001% GO. The decrease in the extractability of the glutenin subunits must be explained by the formation of dityrosine cross-links, since a reducing agent (dithiothreitol) was used in their extraction; and thus all the native disulphide crosslinks or those formed due to the enzyme treatment were reduced to free thiol groups. Therefore the addition of GO led to the formation of high molecular weight aggregates through dityrosine crosslinks. HMW-GS were the most affected fraction likely devoted to the hydrophilic character of its central domain and the hydrophobic character of the N- and C-terminal domains [37], which give them the ability to develop its backbone role in the gluten network [34]. The dityrosine cross-links within

HMW-GS might be formed through linkages of their hydrophobic N- and C-terminal domains.

In the insect infested wheat, HMW-GS are the first to be hydrolysed by the insect proteases [6,39] leading to a structure with higher stability, which could resist the protease hydrolysis [40]. Likely, the resulting protein structure might be formed by HMW-GS bonded to LMW-GS (branches of the backbone) that are highly conserved regions [37]. The present study demonstrated that HMW-GS are also the first glutenin subunits to be cross-linked as a result of the GO treatment. Similar results have been reported when wheat flour proteins were treated with hydrogen peroxide and oxidases [18,36,41] and the formation of macromolecular structures as a consequence of phenolic crosslinkages [42]. The formation of dityrosine cross-links between the glutenin subunits in GO-treated rice has also been described [28].

Results obtained in the present study with damaged wheat agree with previous findings that described the formation of both disulfide and non-disulfide bonds within the albumin/globulin fractions when GO was added, whereas non-disulfide cross-links were formed within the gluten proteins [26].

Effect of glucose oxidase treatment on the thermal behavior of gluten from damaged wheat

The thermal behavior of the gluten proteins studied by using differential scanning calorimetry was determined in order to detect the stability of the structural changes produced by GO in damaged wheat. Only one endothermic peak was observed in the thermograms at 50ºC, which agrees with previous results [33,40,43]. Table 1 summarizes the thermal parameters corresponding to the endothermic peak of gluten. In absence of GO treatment, the peak of damaged gluten appeared at significantly ($P < 0.05$) higher temperatures than that corresponding to sound gluten, but the peak was narrower as indicates the lower denaturation temperature range (ΔTd). The addition of GO at the

minimum level (0.001%) produced a significant ($P<0.05$) enhancement in the onset and peak denaturation temperatures and a significant decrease in the denaturation temperature range. In the presence of increasing GO dosages, the denaturation temperatures (onset, peak and conclusion) of damaged gluten showed a significant ($P<0.05$) decrease, and in consequence a progressive increase of the denaturation temperature range, which was significant till 0.005% GO addition.

The denaturation enthalpy was obtained by integrating the endothermic peaks (Figure 2). Value obtained for damaged gluten without GO treatment was significantly ($P < 0.05$) higher than that for sound gluten[43]. The proteinases mainly affect the backbone formed by the HMW-GS [6], after the insect proteinase hydrolysis the resulting protein structure formed by HMW-GS and LMW-GS has high thermal stability [40]. Kovacs et al (2004)[44] reported that increasing thermal stability is related to higher ratios of monomeric proteins (gliadins and LMW-GS) to HMW-GS, which agree with data obtained in the present study.

The lowest GO dosage produced a decrease in the denaturation enthalpy of damaged gluten. That reduction could be due to the formation of high molecular aggregates between HMW-GS and probably between HMW-GS and LMW-GS, yielding a different protein structure with a thermal stability rather close to sound gluten[43]. When the dosage 0.005% GO was added the thermal stability of damaged gluten was significantly the same to that of sound gluten[43], which might reveal that the thermal stability of the damaged gluten has been completely restored.

Gluten Microstructure determined by SEM

Figure 3 shows the SEM micrographs of untreated gluten from sound and damaged wheat (A,B), and gluten from damaged wheat treated with 0.001% (C), 0.005% (D) and 0.01% (E) GO concentration. Untreated damaged gluten (B) showed a discontinuous network produced as a result of the proteinases injected by bugs that promote the hydrolysis of the covalent bonds between the glutenin subunits. Large and small

fragments of gluten were detected, which did not form part of the continuous network structure. In opposition, the sound gluten showed a nice continuous structure (A).

The treatment of the damaged wheat with the lowest dosage of GO produced a partial recovery of the continuous network structure (C). Bigger fragments, compared to non-treated samples, were observed separately from the whole structure.

The addition of 0.005% concentration of GO produced an evident improvement of the damaged gluten microstructure (D), being rather more difficult to identify small gluten particles without being integrated in the network structure. The treatment of damaged gluten with 0.01% GO (E) resulted in almost a similar structure than that of sound gluten, indicating the recovery of the microstructure of the gluten proteins. Those results agree with the ones obtained in the thermal stability detected by DSC, although for total recovery of the structure 0.01% GO was necessary, whereas for the thermal stability only 0.005% was necessary.

The addition of increasing GO concentrations resulted in the progressive formation of a protein network with a continuous structure that allowed obtaining a gluten microstructure from damaged wheat similar to that from the sound wheat. These results agree with Autio et al (2005)[45] who reported the accumulation of the protein network crosslinked as a result of the TG treatment. This effect was produced because the reinforced protein fibers were not as extended as in the control dough, and were pushed together during the expansion of dough before bread-making. A good relationship was observed between the modifications observed both in gliadins and glutenins by HPCE and the structural changes observed by SEM.

Influence of GO treatment on bread quality evaluation

Figure 4 shows loaves of bread made with non-treated (A) and GO-treated (B) damaged flour as well as bread made with sound flour (C). The addition of GO to damaged flour yielded an improvement in the bread crumb structure, showing more

uniform gas cells than in absence of GO. The effect of the enzyme treatment on the bread technological quality parameters is shown in Table 2.

GO-treated damaged bread reached the same shape index value than non-treated sound bread. That results agree with data obtained by Vemulapalli et al (1998)[22], who reported that GO improved the loaf volume of bread. Nevertheless, in terms of specific volume, the GO treatment produced a significant decrease in that value, likely devoted to an increase in the water retention capacity due to the GO activity. Diverse authors [22,46,47] stated that dough formulated with GO incorporated large amount of pentosans into the insoluble glutenin protein matrix, producing an increase in the water absorption due to the pentosan oxidative gelation.

The gluten hydrolysis caused by the infestation produced a weak gluten network and a weak crumb in bread, but did not affect the cohesiveness, springiness nor resilience. The value of hardness and chewiness were 20% and 25% lower, respectively, than the same values determined for sound bread. The texture profile analysis of the bread obtained from damaged wheat revealed that a significant increase of the values of hardness and chewiness after the GO treatment. Resilience did not change after the enzyme treatment. The cohesiveness in damaged bread is not affected by the enzyme treatment and is similar to sound bread. GO on damaged dough produced the recovering of the values of hardness and chewiness showed by sound bread, and had no effect on the rest of the texture parameters.

The results obtained in the present study agree with data obtained by Vemulapalli et al (1998)[22], who reported the improvement of loaf volume catalysed by GO. Nevertheless, Rasiah et al (2005)[26] reported that the treatment with GO produced improvement in the crumb properties but no increase in product volume. Nevertheless, the increase observed in the shape index and the decrease in the specific volume observed could be ascribed to the higher amount of GO (ten times greater) used by these authors, and to the different flour characteristics.

CONCLUSION

Thermal and electrophoretic studies, as well as bread-making and SEM assays were selected to give a molecular point of view and a eye-visual result of the enzyme treatment. HPCE results demonstrated the higher specificity of GO for gliadins and HMW-GS, which formed high molecular weight aggregates even with the lowest GO dosage. It is supposed that HMW-GS were the most affected maybe devoted to the hydrophobic character of the N- and C-terminal domains that facilitates the aggregation with other subunits. The DSC analysis showed that the high molecular aggregates formed as a result of the GO treatment had a similar thermal stability than the gluten proteins from sound wheat. SEM micrographs supported the results obtained by HPCE and DSC, showing a progressive recovery of the damaged gluten structure, reaching the same appearance than sound gluten when the highest GO level was used.

The bread-making studies confirmed the improved dough functionality of damaged wheat. The activity of GO on damaged wheat flour seems to be very efficient for the recovery of the damaged gluten breadmaking ability.

In summary, the present study shows that appropriate GO dosages could be a good alternative to the use of chemical oxidants by baking industries and farmers, to overcome the detrimental effect of Heteropterous insects infestation on wheat flour.

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FIGURE CAPTIONS

Figure 1: Effect of the addition of increasing GO levels on the total area of gliadins, glutenins, HMW-GS, and LMW-GS of damaged gluten, determined by HPCE. The experimental conditions are detailed in the materials and methods section. Bars describe standard deviation. Means followed by the same letter within a column are not significantly different ($P < 0.05$).

Figure 2: Denaturation enthalpy of the gluten proteins isolated from GO-treated damaged gluten after 30 min incubation at 37ºC. Bars describe standard deviation. Means followed by the same letter within a column are not significantly different ($P < 0.05$).

Figure 3: SEM micrographs of untreated gluten from sound (A) and damaged (B) wheat, and GO-treated gluten from damaged wheat with 0.001% (C), 0.005% (D) and 0.01% (E) GO concentrations.

Figure 4: Bread loaves from non-treated (A) and GO-treated (B) damaged flour, as well as sound flour (C).

Table 1. Effect of GO treatment on the thermal parameters of gluten from insect damaged wheat.

GO (%)	To (ºC)	Tp (ºC)	Tc (ºC)	ΔTd (ºC)
0.000	50.8±0.2c	57.4±0.2c	63.8±0.1c	13.0±0.1b
0.001	52.7±0.9d	59.1±0.9d	64.4±1.0c	11.7±0.1a
0.005	42.6±0.3b	50.3±0.4b	57.5±1.2b	14.9±1.1c,d
0.010	39.4±1.1a	47.9±1.3a	55.1±1.3a	15.8±0.2d

To: onset temperature, Tp: peak temperature, Tc: conclusion temperature, and ΔTd: denaturation temperature range. Values are the mean of four replicates ± standard deviation. Means followed by the same letter within a column are not significantly different ($P < 0.05$).

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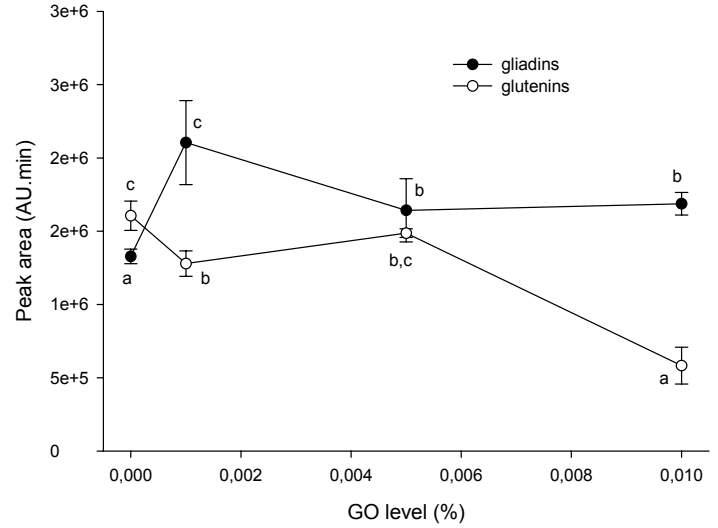
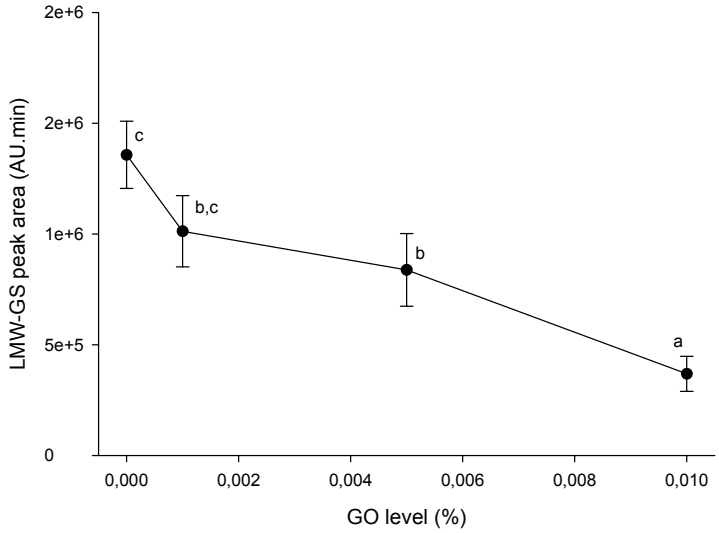
Table 2. Effect of the enzyme treatment on bread quality characteristics and texture parameters.

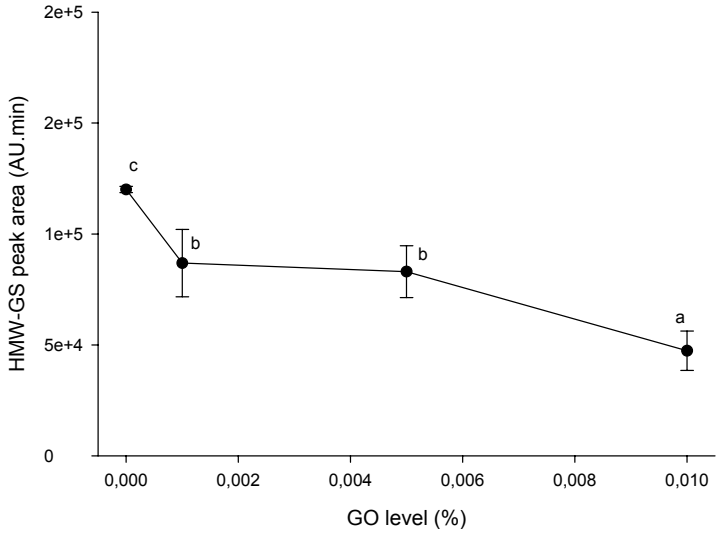
	Sound	Damaged	
GO level (%)	0	0	0.01
Shape Index	1,71±0,07a	1,95±0,09b	1,72±0,03a
Specific Volume (ml/g)	2,59±0,08b	2,54±0,11b	2,36±0,07a
TPA parameters			
Hardness	603,6±62,2b,c	473,7±40,5a	570,2±86,5b
Chewiness	265,2±26,7b	201,7±35,3a	256,6±42,0b
Cohesiveness	0,479±0,009a	0,458±0,053a	0,489±0,017a
Springiness	0,918±0,009a	0,926±0,009a	0,921±0,009a
Resilience	0,193±0,008a	0,171±0,039a	0,191±0,021a

Values are the mean of four replicates ± standard deviation. Means followed by the same letter within a line are not significantly different (P< 0.05).

Figure 1.


gliadins
glutenins
Peak area (AU.min)
3e+6
3e+6
2e+6
2e+6
1e+6
5e+5
0
c
c
b
b
a
b
b,c
a
0,000
0,002
0,004
0,006
0,008
0,010
GO level (%)


LMW-GS peak area (AU.min)
2e+6
2e+6
1e+6
5e+5
0
c
b,c
b
a
0,000
0,002
0,004
0,006
0,008
0,010
GO level (%)


HMW-GS peak area (AU.min)
2e+5
2e+5
1e+5
5e+4
0
c
b
b
a
0,000
0,002
0,004
0,006
0,008
0,010
GO level (%)

2

Figure 2.

2

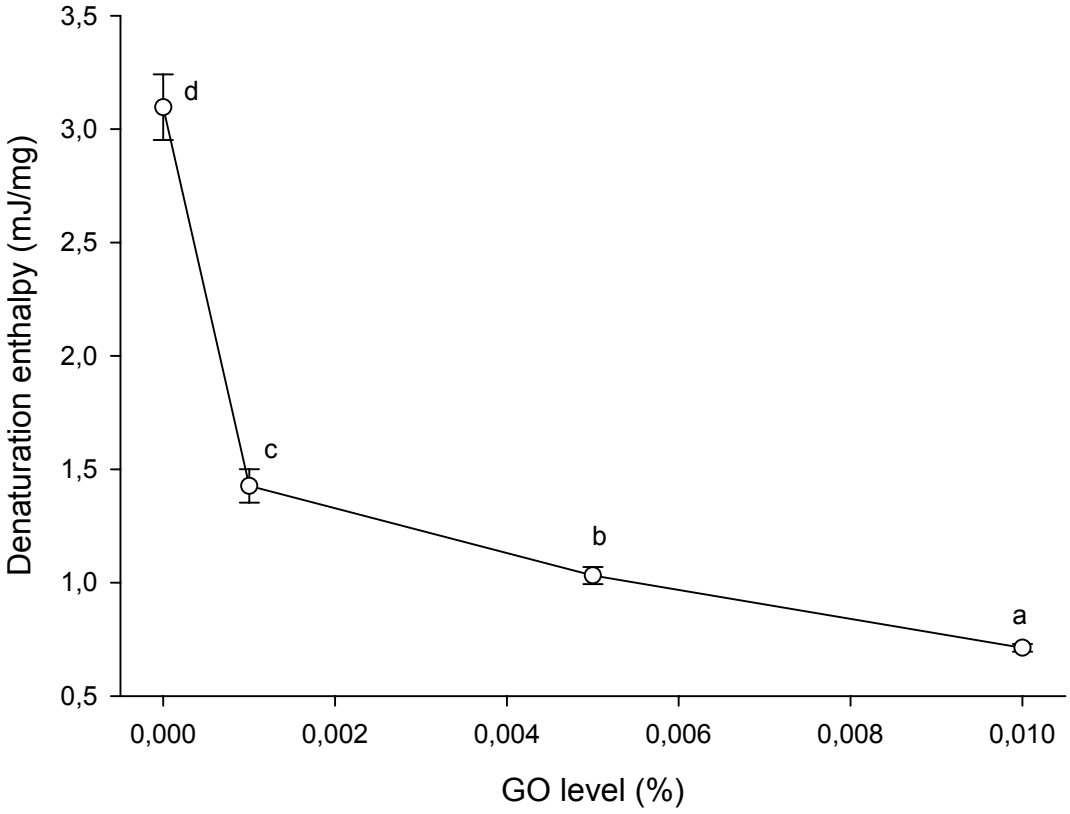

3,5
3,0
2,5
2,0
1,5
1,0
0,5
Denaturation enthalpy (mJ/mg)
d
c
b
a
0,000
0,002
0,004
0,006
0,008
0,010
GO level (%)

Figure 3

2

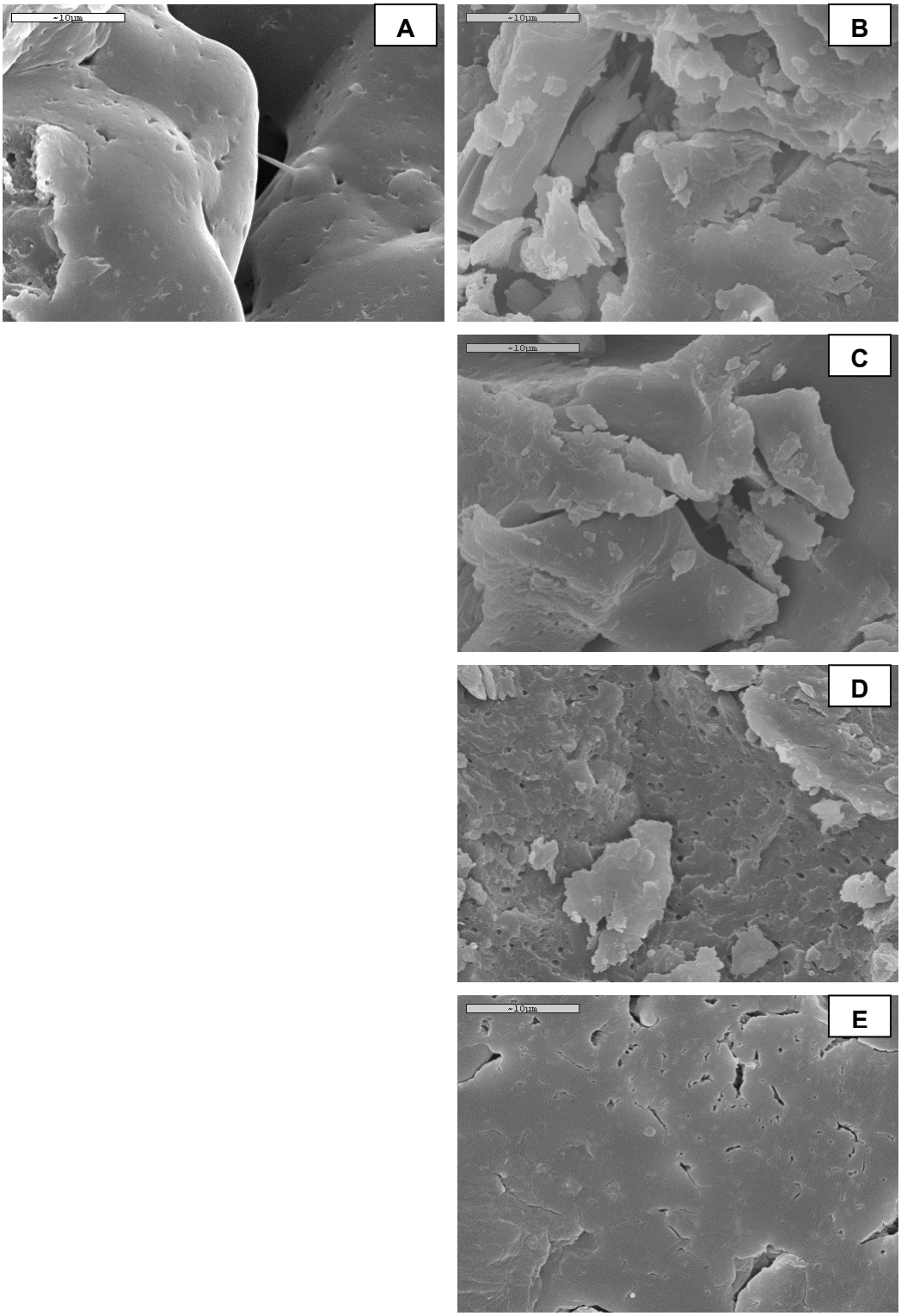


Figure 4