GLUCOSE OXIDASE EFFECT ON DOUGH RHEOLOGY AND BREAD QUALITY: A STUDY FROM MACROSCOPIC TO MOLECULAR LEVEL

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ABSTRACT

Enzymes are used in baking to improve dough handling properties and the quality of baked products. Glucose oxidase (GO) is an enzyme with oxidazing effect due to the hydrogen peroxide released from its catalytic reaction. In this study, the macroscopic effect of increasing glucose oxidase concentrations on wheat dough rheology, fresh bread characteristics and its shelf life during storage was determined. A reinforcement or strengthening of wheat dough and an improvement of bread quality can be obtained with the addition of GO, although inverse effects were obtained when excessive enzyme levels were added. The analysis of the gluten proteins at molecular level by high performance capillary electrophoresis and at supramolecular level by cryo-scanning electron microscopy revealed that the GO treatment modified gluten proteins (gliadins and glutenins) through the formation of disulfide and non-disulfide crosslinks. The high molecular weight glutenin subunits showed to be the most susceptible glutenin fraction to the oxidation action of GO. Excessive addition of GO produced an excessive crosslinking in the gluten network, responsible of the negative effect on the breadmaking properties.

Key words: Glucose oxidase, wheat flour, dough rheology, gluten, proteins, microstructure, bread quality.

INTRODUCTION

Functional properties of bread dough greatly depend on the gluten proteins. In the last years, diverse treatments have been applied for improving the quality of those proteins (Rosell, Wang, Aja, Bean & Lookhart, 2003; Aja, Wang & Rosell, 2003). Protein crosslinking or the formation of covalent bonds between polypeptide chains is a way of modifying the protein functionality and simultaneously increasing its applications in different processes. Oxidation induces the formation of disulfide bonds by coupling of two cysteine residues that are adjacent within a food protein matrix, and dityrosine crosslinks (Tilley, Benjamin, Bagorogoza, Okot-Kotber, Prakash & Kwen, 2001; Rasiah, Sutton, Low, Lin & Gerrard, 2005), in consequence it results the covalent crosslinking of proteins. This reaction on bread dough induces the formation of a protein network with improved viscoelastic and structural properties, and therefore, betters performance for breadmaking (Wikström & Eliasson, 1998; Fayle, Gerrard, Simmons, Meade, Reid & Johnston, 2000). The use of enzymes instead of chemical oxidants is a very interesting option to improve breadmaking performance of dough, because they are perceived as natural and non-toxic food components. Enzymes are specific biological catalysts able to react under mild conditions of temperature and pH, contributing to the formation of covalent bonds between polypeptide chains within a protein (intramolecular crosslinks) or between proteins (intermolecular crosslinks) (Gerrard, 2002).

Oxidative enzymes are increasingly used in bread-making (Poulsen & Hostrup, 1998). Glucose Oxidase (GO) (EC 1.1.3.4) catalyses the oxidation of β-D-glucose to gluconic acid and hydrogen peroxide. The mechanism by which GO improves bread quality is still not completely understood. Diverse authors

(Rosell et al., 2003; Aja et al., 2003; Hoseney & Faubion, 1981; Haarasilta, Pullinen & Vaisanen, 1991; Nakai, Takami Yanaka & Takasaki, 1995; Primo-Martin, Valera & Martinez-Anaya, 2003; Gujral & Rosell, 2004) indicate that hydrogen peroxide produced during GO reaction causes the oxidation of the free sulfhydryl units from gluten protein giving disulfide linkages and the gelation of water soluble pentosans, changing rheological properties of wheat flour dough. This hypothesis was confirmed by Velmulapalli and Hoseney (1998a), who found that free thiol groups of the water soluble proteins of flour or dough decreased in presence of GO. Lately, it has been described the simultaneous formation of dityrosine crosslinks by treating proteins with hydrogen peroxide or peroxidase (Tilley et al., 2001; Singh, 1991; Oudgenoeg et al., 2001). The decrease of the GO effect when there were added free radical scavengers on dough, confirms that hydrogen peroxide is one of the active compounds affecting dough properties (Vemulapalli, Miller & Hoseney, 1998b). Recently, Rasiah et al (2005) stated that the treatment of wheat flour with GO resulted in the crosslinking of water soluble protein (albumin and globulin) fractions, involving both disulfide and non disulfide linkages.

The addition of GO leads to an increase in the elastic and viscous moduli of wheat and rice flour dough (Gujal et al., 2004; Vemulapalli et al., 1998b; Dunnewind, Van Vliet & Orsel, 2002) and also gives less stiff dough than control and its addition has a strengthening effect (Martinez-Anaya & Jimenez, 1997). Primo-Martín et al (2003) concluded that pentosanase/GO combination resulted in dough with improved extensibility yielding better gluten quality. An improvement in the wheat bread loaf volume and crumb grain has been

obtained by adding GO (Vemulapalli et al., 1998b; Xia, Jin & Liang, 1999) and even when it was used in rice flour dough (Gujral et al., 2004). That effect has been attributed to the hydrogen peroxide released from the GO reaction, since Van Oort (1996) found that this compound improved bread volume.

The functional properties of bread dough mainly depend on the proteins forming the gluten network. The objective of this study was to determine the accurate relationship between the effect of increasing concentrations of GO on the macroscopic properties (bread quality and dough rheology) and the molecular composition (gluten proteins and microstructure).

MATERIALS AND METHODS

Materials

Commercial wheat flour (14.2% moisture, 0.49% ash, 12.2 % protein) and instant dry yeast from the local market were used in this study. Glucose oxidase (10000 glucose oxidase units [GU]/g) was kindly supplied by Novo Nordisk (Madrid, Spain). All reagents were of analytical grade.

Dough rheological properties

A consistograph test was carried out in a Consistograph NG (Tripette et Renaud, France) following the AACC Approved Method 54-50 (AACC, 2000). The parameters recorded were: water absorption (WA, water required to yield dough consistency equivalent to 1700 mb of pressure in a constant humidity measurement), and tolerance (Tol, time elapsed since dough consistency reaches its maximum until it decreases down to a 20%). Alveograph test was

118 performed using an Alveograph MA 82 (Chopin, Tripette et Renaud, France) according to the AACC Approved Method 54-30A (2000). The parameters
120 registered were tenacity (P, or resistance to extension), dough extensibility (L), the deformation energy (W), and the curve configuration ratio (P/L).

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Breadmaking procedure

124 A basic bread formula, based on flour weight, was used: 3600 g of flour, water required for obtaining up to 1700 mb consistency, 0.83% (w/w) instant active
126 dry yeast, 2% (w/w) salt, and 0.2% (w/w) sodium propionate. Glucose oxidase (when added) was incorporated to flour at levels of 0.001, 0.005, 0.010 and
128 0.015% (w/w, flour weight basis) before mixing. Dough was optimally mixed until dough development, divided into 315 g pieces, hand-rounded,
130 mechanically moulded, put into well-greased tin pans (measuring 195 x 86 mm), proofed for 90 min at 30ºC and 75% RH, and baked into an electric oven for 35
132 min at 200ºC. Loaves were removed from the pans, cooled for two hours at room temperature, then packed in plastic bags and stored at 25ºC for aging
134 studies.

Bread quality analysis was carried out by measuring weight, volume
136 (determined by seed displacement in a loaf volume meter), specific volume, and height/width ratio of the central slice. Crumb hardness was measured in a
138 Texture Analyzer TA-XT2i (Stable Microsystems, Surrey, UK) equipped with an aluminium 25 mm diameter cylindrical probe. Slices of 2 cm thickness were
140 compressed to 50% of their original height at a crosshead speed of 1 mm/s. The resulting peak force of compression was reported as hardness. Bread

142 hardness was measured over twelve-day period of storage. Three replicates from three different sets of baking were analysed and averaged.

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Effect of GO on gluten composition and structure

146 In order to guarantee a good distribution of the enzyme on the dough, flour and GO were mixed during one hour using a Rotary Mixer MR 2L (Chopin, Tripette 148 et Renaud, France). 10g of wheat flour treated with different GO dosages were used for extracting gluten proteins, following the AACC Approved Method 38- 150 12A (AACC, 2000) by using the Glutomatic (Perten, Stockholm, Sweden). Wet gluten balls were then freeze dried for further characterization.

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A sequential protein extraction of gliadins and glutenins from freeze-dried gluten 154 samples was made following the method previously described (Bean, Bietz & Lookhart, 1998). High molecular weight glutenin subunits (HMW-GS) and low 156 molecular weight glutenin subunits (LMW-GS) were also isolated (Bean & Lookhart, 2000). Samples were extracted in duplicate. Electrophoretic 158 separations of the proteins were made using a Beckman MDQ instrument. Uncoated fused silica capillaries (Composite Metal Services Ltd, Worcester, 160 UK) of 50 μm i.d. x 27 cm (20 cm L_D) were used for all separations. Protein electrophoresis was performed with 50mM iminodiacetic acid (IDA) in 162 acetonitrile: hydroxypropylmethylcellulose: water (20:0.05:79.95, v/v) at 45ºC and 30 kV, the optimum separation conditions described by Bean and Lookhart 164 (2000).

166 Microstructure of gluten and glutenins was studied by cryo-scanning electron microscopy (Cryo-SEM), and gliadins by scanning electron microscopy (SEM). 168 Gluten was manually extracted according to the ICC (1984) method. Gliadins were extracted from gluten by ultracentrifugation (Medifriger – BL, Selecta 170 ultracentrifuge) in a solution 50 % (v/v) of propanol. Three extractions (10,000 rpm, 15 min) and two washes (10,000 rpm, 5 min) were done with each sample. 172 The supernatant containing the gliadins was freeze-dried in a Telstar Lioalfa 6 lyophiliser. The pellet obtained was assumed as the glutenins fraction. The 174 study of gluten and glutenins was performed with a cryostage equipment CT-1500C (Oxford Instruments) coupled to a scanning electron microscope Jeol 176 JSM-5410. Samples were frozen by immersion in slush nitrogen (below –210ºC) and rapidly transferred to a cryostage at 1kPa. After that, samples were 178 freeze-fractured and gold coated at vacuum (0,2 kPa), with an ionization current of 2mA. Thus, the fractured surface was directly observed while it was 180 maintained at 15kV and temperature below –130ºC. The freeze-dried gliadins were mounted directly on stubs and coated with gold with a 35 mA current in a 182 sputter coater for 1 min. Then, they were observed with a JEOL JSM-6300 scanning electron microscope with an accelerating voltage of 10 kV.

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Statistical analysis

186 In order to assess significant differences among samples, a multiple comparison analysis of samples was performed using the program Statgraphics 188 Plus 5.1. Fisher's least significant differences (LSD) test was used to describe means with 95% confidence.

190

RESULTS AND DISCUSSION

Effect of enzyme treatment on dough rheology

Rheological properties of wheat dough containing different amounts of glucose oxidase are summarized in Table I. The addition of GO did not significantly ($P<0.05$) modify the water absorption, regardless when 0.005% GO was added that produced a significant ($P<0.05$) increase of the WA. Vemulapalli, Miller and Hoseney (1998) observed a drying effect on dough when adding glucose oxidase. Dough tolerance (Tol) showed a significant ($P<0.05$) enhancement when added the highest GO concentration. Thus the addition of GO promotes an increase in dough stability when overmixing. This result agrees with the increased relaxation time reported by Wikström and Eliasson (1998).

The most dramatic effect of GO addition was observed when biaxial properties of wheat dough were assessed in the alveograph. GO induced a significant ($P<0.05$) modification of the alveograph parameters. The addition of the lowest GO concentration (0.001%, w/w) did not significantly modified dough tenacity (P), but higher concentration resulted in a steady increase of the tenacity besides to a significant decrease ($P<0.05$%, w/w) in dough extensibility (L). It should be noted that the effect on extensibility seems to reach a maximum at 0.010% (w/w) GO concentration and no further significant decrease was observed at higher GO concentration. Overall effect on tenacity and extensibility led to a significant ($P<0.05$) increase of the curve configuration ratio (P/L). Deformation energy (W) steadily increased when adding increasing enzyme amounts, being significantly ($P<0.05$) affected at GO concentration higher than 0.005% (w/w). These results corroborate the previous ones obtained when dough properties were studied with uniaxial test, where less extensible and

more resistant dough were obtained in the presence of GO (Poulsen et al., 1998; Primo-Martin, Wang, Lichtendonk, Plijter & Hamer, 2005). The strengthening effect of GO on the wheat dough has been attributed to the formation of additional protein crosslinks via disulfide and maybe phenolic linkages (Rosell et al., 2003, Primo-Martin et al., 2003; Gujral et al., 2004), as well as the oxidative gelation of water- soluble pentosans (Vemulapalli et al., 1998b; Crowe & Rasper, 1988).

Effect of enzyme treatment on bread quality

As can be seen in Table II, the addition of GO induced different effect on bread specific volume depending on the enzyme concentrations. The addition of the low GO concentrations (0.001-0.005%, w/w) yielded loaves with significant ($P<0.05$) greater specific volume and better shape (as indicates the height / weight ratio). This behaviour came accompanied by a decrease in the crumb hardness of breads and indicated an improving effect also in their crumb grain. These results agree with previous findings of Vemulapalli, Miller and Hoseney (1998) when similar breadmaking process was carried out. They explained this behaviour due to the improvement of baking performance promoted by the addition of oxidants to weak flours. In fact, this effect was even more marked when GO was added to gluten free cereals, which do not originally develop a protein network (Gujral et al., 2004).

Conversely, when higher GO concentrations (>0.005%, w/w) were added no significant ($P<0.05$) effect on the quality parameters (crumb hardness and specific volume) was observed. This result are in accordance with those of

240 Rasiah et al. (2005), who do not observed differences between loaf volume in the presence or absence of GO.

242

Effect of enzyme treatment on evolution of bread quality during its
244 **storage**

Bread crumb remained especially soft in the 0,001% GO treated bread during
246 its storage at 25°C (Figure 1). Differences were even more evident after a storage period of twelve days. This result could partially be ascribed to the
248 higher initial specific volume of bread and in consequence lower crumb hardness, and the difference increase during storage. At this enzyme
250 concentration, the plot of crumb firmness vs. storage time showed lower hardness increase over the storage period, suggesting an antistaling effect of
252 the GO. Primo-Martin et al (2003) stated that dough formulated with GO resulted in a large amount of total pentosans associated with glutenin
254 macropolymer (GMP) due to the incorporation of pentosans into the insoluble glutenin protein matrix. This effect linked to the ability of pentosans to retain
256 high amounts of water through interchain associations involving oxidative coupling and chain entanglements (Gujral et al., 2004) might be responsible of
258 the reduced hardness increase.

The positive effect observed during storage decreased with the increasing of
260 enzyme concentration, leading even harder crumbs than the control when GO concentrations higher than 0.005% (w/w) were added. The negative effect
262 promoted by higher GO levels might be due to an over-oxidizing effect on the proteins and an intense gelation of water-soluble pentosans produced by
264 hydrogen peroxide action (Vemulapalli et al., 1998b).

Effect of enzyme treatment on gluten proteins

In order to understand the effect of GO at molecular level, proteins were sequentially extracted and gluten storage proteins (gliadins and glutenins) and the glutenin subunits (HMW-GS and LMW-GS) were analyzed by HPCE. Following the method reported by Bean, Bietz and Lookhart (1998), gliadins were extracted under non-reducing conditions, whereas reduced conditions were used for extracting glutenins.

No significant differences were observed in the area beneath the gliadin and total glutenin curves when concentrations of GO up to 0.005% were added, only at the highest GO concentration tested (0.010%, w/w) (Figure 2). The same trend was observed in the LMW-GS peak areas (Figure 2), showing a significant ($P<0.05$) decrease at the highest GO concentration. In the case of the HMW-GS, significant ($P<0.05$) differences were readily detected at lower concentration of enzyme (0.005%, w/w), thus minor GO amount was enough to modify these subunits.

Initially, Vemulapalli and Hoseney (1998) described that, based on the quantification of SH content, GO induced the oxidation of the water soluble proteins and did not directly modify gluten proteins. The addition of glucose oxidase produces a crosslinking of the albumins and globulins, and that effect involves disulfide crosslinking but predominantly non-disulfide crosslinkages (Rasiah et al., 2005). In the case of gliadins, Rasiah et al (2005) did not found any modification of this fraction in the presence of glucose oxidase , as happen in the present study when GO concentrations were lower than 0.010% (w/w). Likely, due to the compact and symmetrical structure of the gliadins, high levels

290 of GO are necessary to induce a significant decrease of them. Regarding glutenins extracted under reducing conditions, the addition of glucose oxidase
292 at the highest amount tested induced a number of non-disulphide crosslinks yielding polymeric structures with reduced extractability, in agreement with
294 findings of Rasiah et al. (1998). In order to support the electrophoretic results, the microstructure of the gliadins and glutenins was analyzed by SEM and cryo-
296 SEM, respectively (Figure 3). Micrographs of the gliadins (Figure 3 A) showed their structure as spherical particles of 0.5μm diameter forming agglomerated
298 structures. After the treatment with 0.010% (w/w) GO (Figure 3 B), the size of the particles increased from 0.5 μm up to approximately 2.0 μm. In the case of
300 the glutenins (Figure 3 C), the micrograph revealed a network structure that upon GO treatment displayed a weaker and more open structure, although still
302 showing continuous arrangements (Figure 3 D). The microstructure of the gliadins and glutenins agree with previous observations of Lindsay and Skerrit
304 (1999) that describe the network structure of the glutenins and the space filling role of the gliadins uniformly dispersed within gluten strands. The H_2O_2 released
306 from the GO reaction induced the formation of both disulfide and non-disulfide crosslinks (Rosell et al., 2003; Aja et al., 2003; Hoseney et al., 1981; Haarasilta
308 et al., 1991; Nakai et al., 1995; Primo-Martin et al., 2003; Gujral et al., 2004), leading larger gliadin agglomerates and promoting a weaking effect on the
310 glutenin network structures, and in consequence the modification of dough viscoelastic properties previously described. The formation of disulfide
312 crosslinks between HMW-GS and LMW-GS of wheat gluten as a result of the application of an oxidative agent (ascorbic acid) has been widely described by
314 diverse authors (Grosch & Wieser, 1999; Koehler, 2003), and recently the

formation of non-disulfide crosslinks induced by the presence of GO (Rasiah et al., 2005).

When the polimerisation kinetic of the HMW-GS and LMW-GS was analysed, it showed that the decrease of the protein fraction extractability comparing untreated and treated samples with the highest GO concentration was 39.9% for LMW-GS and 45.4% for HMW-GS. Those results demonstrate that the effect of the GO treatment on the glutenin fraction is mainly directed to the HMW-GS, indicating that they are more prone to form non-disulfide crosslinks, which could be of dityrosine nature.

Effect of GO treatment on dough microstructure

The objective of the microstructure analysis was to elucidate the relationships between dough handling/baking properties and food structure as suggested by Autio and Laurikainen (1997). Gluten without enzyme treatment observed by Cryo-SEM showed a compact closed structure (Figure 4A). Higher magnification micrograph (Figure 4B) showed a detailed image of untreated gluten like a continuous protein network, with a uniform distribution of the absorbed water through its structure. The addition of small amount of GO (0.001%, w/w) increased the number of pores and its size (Figure 4C), resulting a network with higher density of proteins than untreated gluten. It seems that apparently GO has reinforced the protein-protein interactions, if it is compared with untreated gluten, yielding a coarser and less oriented gluten fibrils (Figure 4D). The addition of ten times higher GO concentration (0.010%, w/w) induced a gluten network with a more discontinuous protein matrix structure, loosing

completely its original orientation (Figure 4F). The number and size of the pores greatly increased in comparison with previous micrographs, and even some pores got stacked giving a disrupted-like structure (Figure 4E). This structure had an irregular ability to retain water, with water-rich zones showing a typical eutectic formation, generated by the sublimation process produced during the sample preparation for Cryo-SEM observation. The gluten matrix produced after the treatment with the highest GO dosage was less uniform and likely with poor ability to hold the gas released during the proofing process (Berglund, Shelton & Freeman, 1991), which agreed with the bread quality results.

Microscopy observations together with the results obtained in the gluten proteins characterization indicated that GO action would intensify the protein-protein interactions with a dosage of 0.001% (w/w). However, at the highest GO concentration (0.010 %), the disrupted-like structure observed in the micrographs of the gluten treated would be related with the modification of the wheat proteins detected as a significant decrease on the amount of the HMW-GS and LMW-GS. The higher susceptibility to the oxidation showed by HMW-GS in comparison with LMW-GS, may reinforce the gluten backbone structure of gluten, yielding bigger pores and coarser gluten fibrils. Therefore, higher enzyme concentration (0.01%, w/w) would induce an over-oxidizing effect with a reduction in the glutenins and gliadins fractions and with negative consequences in the protein-protein and protein-water interactions.

CONCLUSION

The addition of glucose oxidase to wheat dough produces an important modification on the gluten proteins related with the formation of high molecular

weight polymers that reinforced the gluten network. This agrees with the coarser and nonuniform gluten fibrils forming the protein matrix structure observed by Cryo-SEM, and the decrease of extractable LMW-GS and HMW-GS observed by HPCE. HMW-GS showed to be the most susceptible glutenin fraction to the oxidation and the formation of non-disulfide bonds.

The addition of increasing GO concentrations to wheat flour dough produced significant changes on dough rheology and bread quality. The extent of the effect is highly dependent on the amount of enzyme and the original wheat flour quality. Electrophoretic results and microscopy observations show that high GO amount over-reinforce the gluten network that will retain gas poorly. Despite some types of deficiencies in breadmaking quality of wheat flour could be overcome by GO treatment, it should be stressed that an over-dosage yields a detrimental effect on the handling characteristics of dough and the quality of the resulting bread.

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FIGURE CAPTIONS

480

Figure 1: Effect of increasing levels of GO on crumb hardness during bread
482 storage at 25°C. Bars describe the standard deviation. Legend indicates the enzyme concentration (%, w/w flour basis).

484

Figure 2: Effect of GO dosages on the total area of gliadins, glutenins, HMW-
486 GS and LMW-GS determined by HPCE. The experimental conditions are detailed in the materials and methods section. Bars describe the standard
488 deviation. Different letters within series indicate significant ($P<0.05$ differences).

490 **Figure 3**: Cryo-SEM micrographs of gliadins (A and B) and glutenins (C and D) extracted from untreated dough (A and C), and from dough treated with 0.010%
492 (w/w) of glucose oxidase (B and D).

494 **Figure 4**: Cryo-SEM micrographs of gluten extracted from the untreated dough (A and B), from dough treated with 0.001% (w/w) (C and D) and 0.010% (w/w)
496 of GO (E and F).

Table I. Rheological properties of dough containing increasing concentrations of GO.

GO dosage (%, fb)	WA (%)	Tol (sec)	P (mm)	L (mm)	W (x 10^{-4} J)	P/L
0	55.1a	123.3a	41a	95.5d	115.5a	0.43a
0.001	56.4ab	127.0a	37a	110.5c	124.5ab	0.34a
0.005	56.5b	148.0ab	57b	71.0b	124.0ab	0.81b
0.010	56.1ab	151.0ab	72c	52.0a	134.0b	1.41c
0.015	55.7ab	164.5b	85d	45.5a	150.0c	1.88d

498 GO: glucose oxidase, fb: flour basis, WA: water absorption, Tol: tolerance, P: tenacity, L: extensibility, W: deformation energy, P/L: curve configuration ratio.
500 Means within columns followed by the same letter were not significantly different ($P<0.05$).

502

Table II. Bread quality of dough containing increasing concentrations of GO.

GO dosage (% fb)	Specific volume (cm^3/g)	Height/Width ratio	Crumb Hardness (g)
0.000	3.46a	0.73a	726.3c
0.001	4.42c	0.99c	310.7a
0.005	3.72b	0.83b	604.2b
0.010	3.41a	0.78ab	762.0c
0.015	3.44a	0.81b	763.3c

504 Fb: flour basis. Means within columns followed by the same letter were not significantly different ($P<0.05$).

506 Fig. 1

508

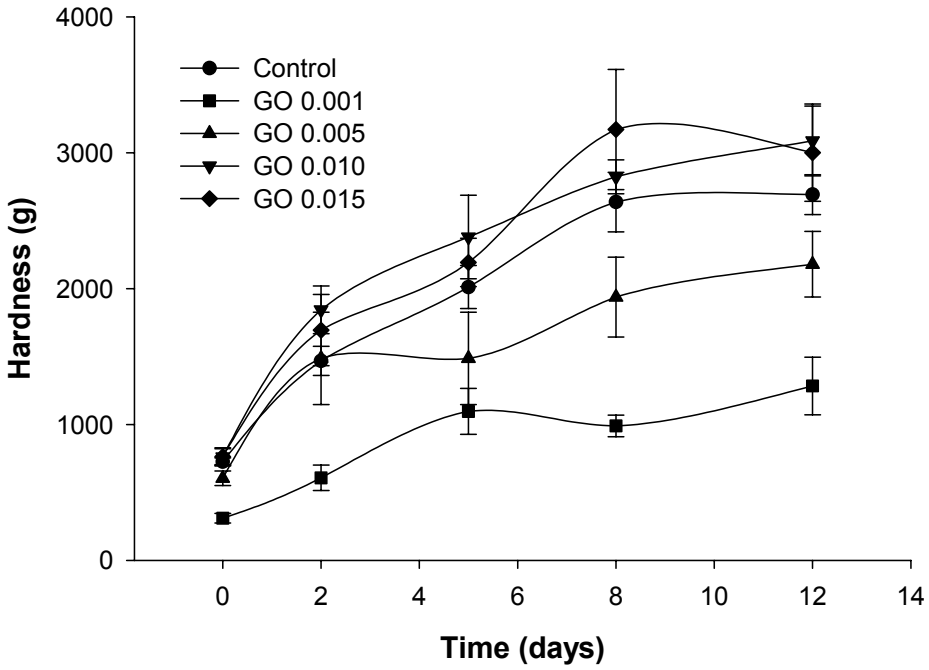
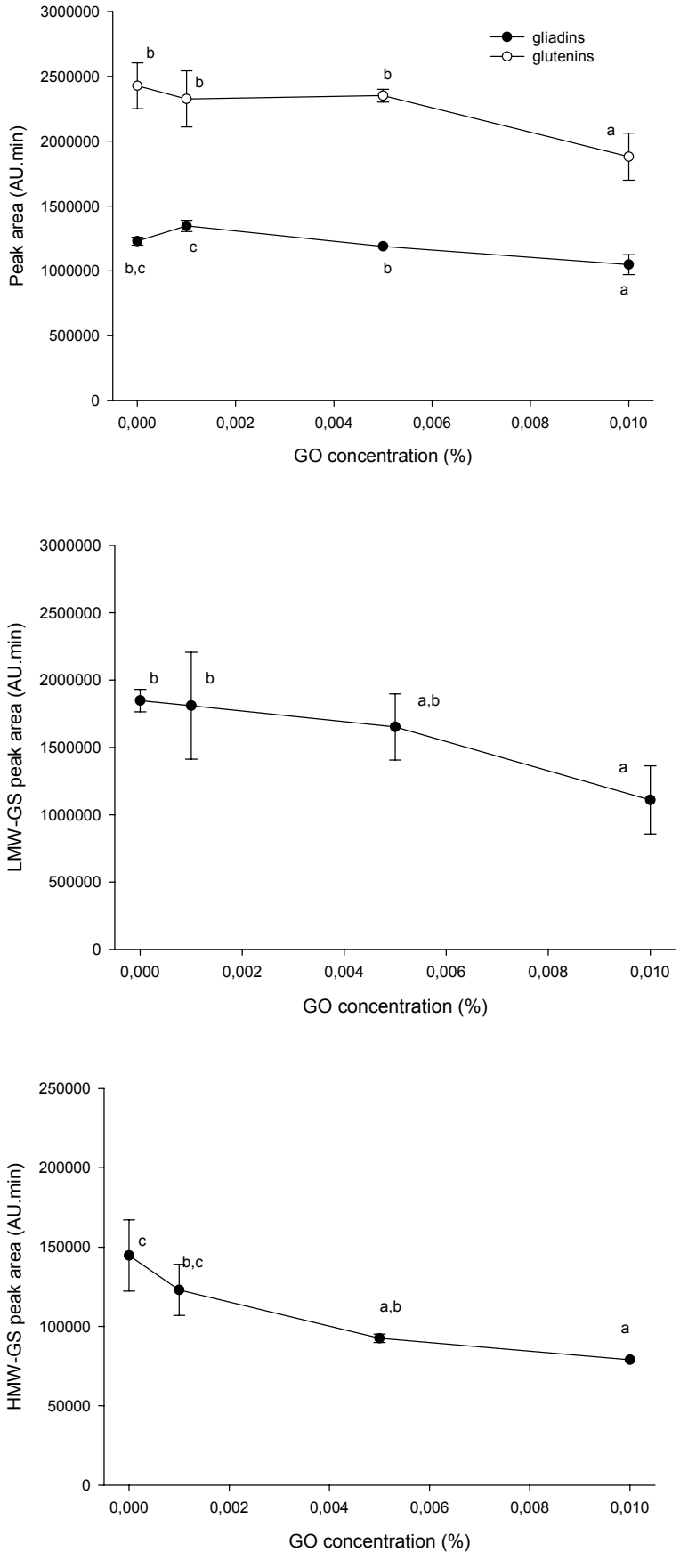
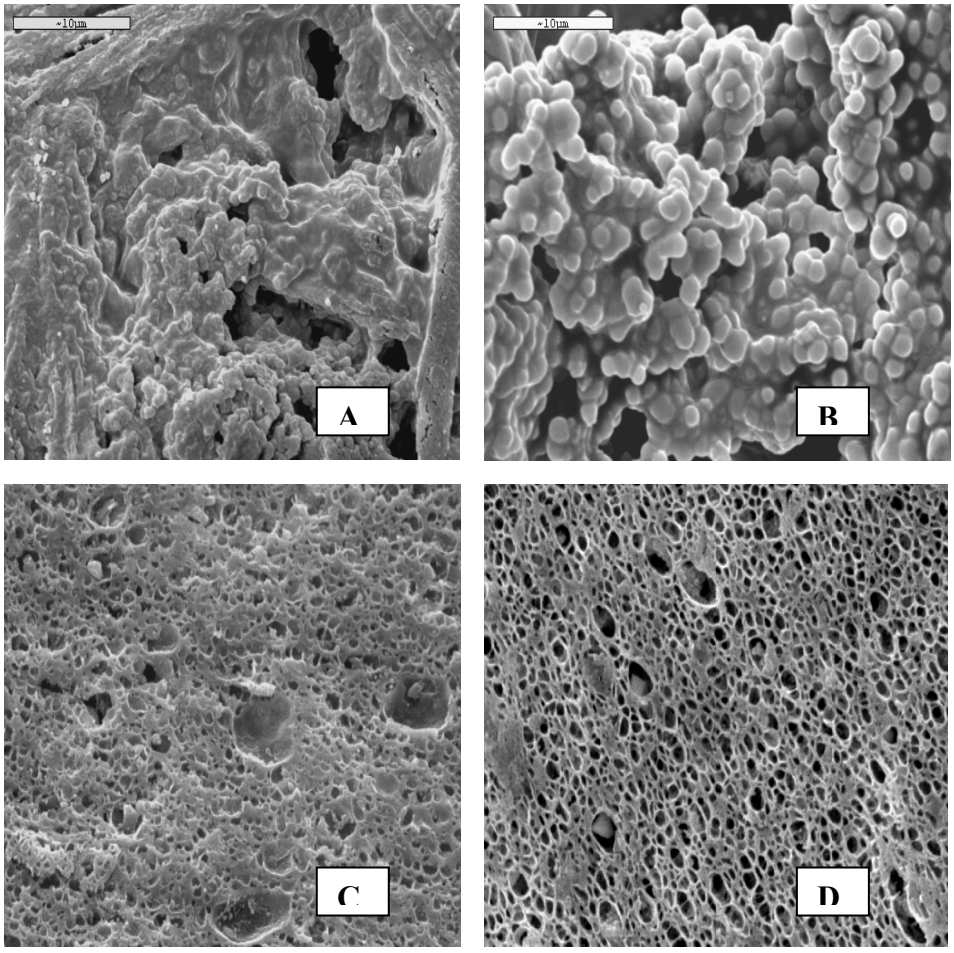

4000
3000
2000
1000
0
Hardness (g)
Control
GO 0.001
GO 0.005
GO 0.010
GO 0.015
0
2
4
6
8
10
12
14
Time (days)

Fig. 2

510


gliadins
glutenins
Peak area (AU.min)
3000000
2500000
2000000
1500000
1000000
500000
0
b
b
b
a
b,c
c
b
a
0,000
0,002
0,004
0,006
0,008
0,010
GO concentration (%)
LMW-GS peak area (AU.min)
b
b
a,b
a
GO concentration (%)
HMW-GS peak area (AU.min)
250000
200000
150000
100000
50000
0
c
b,c
a,b
a
GO concentration (%)

512 Fig. 3



514

Fig. 4