IMPROVEMENT OF DOUGH RHEOLOGY, BREAD QUALITY AND BREAD SHELF-LIFE BY ENZYMES COMBINATION

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Abstract

Present work seeks to systematically analyse the individual and synergistic effects of some gluten-crosslinking enzymes (transglutaminase, glucose oxidase and laccase), along with polysaccharide and gluten degrading enzymes (alpha-amylase, xylanase and protease), in breadmaking systems. Except glucose oxidase (GO) and laccase (LAC), enzymes affected significantly to viscoelastic properties of dough. Results confirmed the strengthening effect exerted by transglutaminase (TG). However, alpha-amylase (AMYL), xylanase (XYL) and protease (PROT) promoted a similar decrease in all dynamic moduli analysed, particularly after 180 min of incubation. Addition of XYL to TG containing samples showed to be an interesting alternative to prevent excessive dough strengthening. Bread quality parameters were significantly affected by individual enzyme addition, except when LAC was used. TG diminished loaf specific volume and provided a finer crumb structure. Polysaccharide degrading enzymes and PROT led to better shape, greater specific volume and void fraction of loaves. Significant interactions between TG and all the other enzymes except GO, were proved. According to crumb texture evolution during storage, bread staling increased with TG addition, whilst AMYL, XYL and PROT exhibited a significant antistaling effect.

Key words: Enzymes, wheat flour, dough rheology, bread quality, bread staling.

Introduction

The breadmaking process and the quality of the product depend, to great extent, on the ingredients used, that is flour, yeast, salt and water. Moreover, a variety of additives are used to improve dough formulation, dough machinability, process tolerance, and bread quality. At the beginning of 80´s decade, the use of enzymes of microbial origin became increasingly important as an interesting alternative to chemical improvers. Nowadays, a wide range of enzymes produced especially for bread-making is available for bakers.

Breadmaking quality of wheat flour is largely determined by the quantity and quality of its proteins. During dough mixing, wheat flour is hydrated and the gluten proteins are transformed into a continuous cohesive viscoelastic gluten protein network. In this context, gluten-crosslinking enzymes can actively contribute to confer the functional properties to dough. Transglutaminase (TG; protein-glutamine gamma-glutamyltransferase) (EC 2.3.2.13) has been reported extensively for its ability to crosslink different food proteins (Kuraishi, Yamazaki, & Susa, 2001; Motoki & Nio, 1983; Motoki & Seguro, 1998; Zhu, Rinzema, Tramper, & Bol, 1995). When it is used in breadmaking, TG is able to improve the functionality of flour proteins through the formation of large insoluble polymers (Larré, Denery, Popineau, Deshayes, Desserme, & Lefevre, 2000; Bonet, Caballero, Gómez, & Rosell, 2005; Caballero, Bonet, Rosell, & Gómez, 2005). High molecular weight (HMW) glutenins are the most affected protein fraction (Bauer, Koehler, Wieser, & Schieberle, 2003a; Gerrard, Fayle, Brown, Sutton, Simmons, & Rasiah, 2001; Larre et al., 2000; Rosell, Wang, Aja, Bean, & Lookhart, 2003), but low molecular weight (LMW) glutenins (Autio, Kruus,

Knaapila, Gerber, Flander, & Buchert, 2005), α-gliadin (Bauer et al., 2003a) or even water extractable albumins and globulins (Gerrard et al., 2001) have been also proposed as substrates for TG.

Disulphide bonds are the most prominent linkages in biology and play an important role during the mixing of wheat flour and water to generate dough (Gerrard, 2002). Oxidative enzymes have a strong impact on the dough thiol-disulphide system and hence, on the properties of the dough (Goesaert, Brijs, Veraverbeke, Courtin, Gebruers, & Delcour, 2005). Glucose oxidase (GO) (EC 1.1.3.4) is the currently preferred enzyme alternative to chemical oxidizing agents for bread improvement (Poulsen & Hostrup, 1998; Bonet, Rosell, Caballero, Gomez, Pérez-Munuera, & Hernando, 2006). The hydrogen peroxide produced during GO reaction promotes the formation of disulfide linkages in gluten protein and the gelation of water soluble pentosans (Gujral & Rosell, 2004a; Hoseney & Faubion, 1981; Primo-Martin, Valera, & Martínez-Anaya, 2003; Vemulapalli & Hoseney, 1998). Laccase (LAC; p-diphenol oxygen oxidoreductase) (EC 1.10.3.2) is another oxidative enzyme which recently has attracted a considerable interest in breadmaking. LAC catalyses the oxidative gelation of feruloylated arabinoxylans by dimerization of their ferulic esters (Figueroa-Espinoza, Morel, & Rouau, 1998; Labat, Morel, & Rouau, 2001). Through the aforementioned mechanisms, gluten-modifying enzymes may produce beneficial effects during breadmaking, affecting positively to rheological behaviour of dough and the quality of final product. Additionally, their association with different enzyme principles have been proposed (Bollaín & Collar, 2004; Caballero, Gómez, & Rosell, 2006; Collar & Bollaín, 2004, 2005b). Due to their active contribution to fresh quality enhancement and/or staling

prevention of bakery products, polysaccharide-degrading enzymes have been usually used for these aims. Among them, amylases (and concretely alpha-amylase) and pentosanases are some of most representative. However, reports on the combined use of strengthening enzymes are limited. On the other hand, these enzymes act on different protein fractions (glutenins, gliadins, albumins or globulins) according to their particular action mechanism, affecting in different way to the functional properties of bread dough. Present work seeks to be a systematic study for analysing the individual and synergistic effects of gluten cross-linking enzymes in breadmaking systems. In order to improve their response, the effect of the aforementioned enzymes was evaluated in combination with polysaccharide and gluten-degrading enzymes (alpha-amylase, xylanase and protease). Rheological behaviour of dough, fresh pan bread volume, shape, texture and crumb grain characteristics, as well as the rate of bread staling were analysed for assessing the effects of enzyme treatments.

Materials and methods

Materials

A commercial blend of wheat flours provided by Harinera Castellana (Medina del Campo, Spain) was used in this study (Table 1). Six commercial enzymes were used: a glucose-oxidase [Gluzyme Mono 10000 BG (GO)], containing 10000 glucose oxidase units/g, a pentosanase [Pentopan Mono BG (XYL)] containing 2500 fungal xylanase units/g, a laccase [NZ 27011 (LAC)] containing 10500 phenol oxidase units/g, an amylase [Fungamyl SG (AMYL)] containing 2500 fungal amylase units/g, a protease [Flavourzyme 1000 L (PROT)]

containing 1000 aminopeptidase units/g (all of them from Novozymes, Denmark), and transglutaminase (Microbial TGM Activa WM, TG) containing 100 transglutaminase units/g, manufactured by Ajinomoto Co. Inc. (Tokyo, Japan).

Instant dry yeast and salt employed in breadmaking process were obtained from the local market. All chemicals used for analyses were of analytical grade.

Dynamic rheological test.

Selected dosages of the enzymes GO, XYL, LAC, AMYL, PROT and TG were added following the supplier's recommendations, 3 mg, 6 mg, 20 μl, 1 mg, 5 μl and 500 mg/100 g of flour respectively. Enzymes were added according to the experimental design showed in Table 2. All of them were tested at two levels: 0 (absence of enzyme) and 1 (presence of enzyme at recommended dose). Flour and enzymes (when added) were mixed during one hour before the tests, using a Rotary Mixer MR 2L (Chopin, Tripette et Renaud, France).

Dough was prepared by mixing flour-enzyme blends with the water [52.8 % (w/v), flour basis] in the Alveograph mixer, according to procedure summarized in the AACC standard method 54-30 (AACC, 2000). After mixing, dough was extruded and cut with a knife-spatula in three pieces that were placed between two glass plates. The pieces were sheeted to a thickness of 5 mm and cut using a circular 54 mm diameter cutter. The resulting pieces were placed in the resting compartment of the Alveograph at 25 ºC, and kept for different resting periods (30, 60 and 180 min), before testing in the dynamic rheometer.

Dynamic rheological analysis was performed using a controlled stress rheometer (RheoStress 1, Thermo Haake, Karlsruhe, Germany) with parallel plate geometry (60 mm diameter). The dough was placed between parallel plates, the gap adjusted to 3 mm and the excess dough removed. To prevent drying at the edges, a thin layer of vaseline oil was applied to cover the exposed dough surfaces. Before measurements, doughs rested for 5 min, to allow relaxation after sample handling. To determine the linear viscoelastic region of the dough, dynamic moduli were collected and plotted as a function of the applied stress.

Oscillatory tests with a frequency sweep from 0,1 to 100 Hz were conducted at a constant stress of 5 Pa at 25 ºC. The dynamic rheological properties of samples were assessed by the storage modulus G´ (elastic modulus) and the loss modulus G˝ (viscous modulus). The complex modulus (G*) that represents the resistance of dough to deformation or the total energy needed to induce changes in the samples was calculated as $G^* = (G'^2 + G''^2)^{1/2}$. To detect significant differences among enzyme treatments, the values of dynamic moduli obtained at a frequency of 1 Hz were used (Caballero et al., 2005; Martínez-Anaya & Jimenez, 1997).

Breadmaking procedure.

Dough formulation, based on 100 g flour, included: 57 mL water, 2 g salt, 0.83 g instant active dry yeast, 0.2 g sodium propionate and the amount of enzyme indicated previously for each sample. Dough was optimally mixed (14 min), divided into 315 g pieces, hand-rounded, mechanically moulded, put into well-greased tin pans (measuring 195 x 86 mm), and proofed for 90 min at 30ºC and

75% RH. The pieces were baked into an electric oven for 35 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 studies.

Evaluation of bread quality.

Quality analysis of fresh bread samples was carried out by measuring weight, volume (determined by seed displacement in a loaf volume meter), specific volume, and height/width ratio of the central slice.

Crumb texture was determined by a Texture Analyzer TA-XT2i (Stable Microsystems, Surrey, UK) provided with the software “Texture Expert”, and equipped with an aluminium 25 mm diameter cylindrical probe. Slices of 2 cm thickness were compressed to 50% of their original height in a “Texture Profile Analysis” double compression test (TPA), at 1 mm/s speed test, with a 30 s delay between first and second compression. Primary parameters [hardness (gram-force, gf), cohesiveness, springiness and resilience] and secondary mechanical characteristics [gumminess (gf) and chewiness (gf)] were calculated from the TPA graphic. Bread texture was measured over twelve-day period of storage.

Crumb grain characteristics of bread were assessed using a digital image analysis (DIA) system. Images were previously acquired at 300 dots per inch (0.0843 mm/pixel) with a 1236USB Artec scanner (Ultima Electronics Corp., Taiwan). The analysis was performed on 41 x 41 mm squares taken from the centre of the slice. This field of view represented approximately one-third of the cross-sectional area of the loaves. Images were processed using Leica QWin

Pro V3.1 software (Leica Microsystems Imaging Solutions Ltd., UK). A cluster analysis method commonly known as the "K-means algorithm" was used to obtain, for each bread slice examined, an optimum gray level threshold to divide images into regions of cells and surrounded cell wall material (Sapirstein, 1999). Subsequent to cell detection, feature extraction was performed for each bread slice analysed. The crumb grain characteristics studied were: crumb brightness (mean gray level), mean cell area (mm^2), cell density ($cells/cm^2$; higher levels denote finer structure), cell to total area ratio (or void fraction, computed as the percentage of the total analysed square occupied by detected cells), mean cell wall thickness (mm; calculated as the averaged mean intercellular distance of neighbouring cells sampled) and crumb grain uniformity (computed as the ratio of number of small to large cells using a cell area threshold of 4.0 mm^2. Larger values denote a more uniform cellular structure) (Sapirstein, 1999).

Statistical analysis

Experimental design was conducted by means a 2-level half-fractional factorial design in order to evaluate all single effects and second order interactions between factors. Resultant design is shown in Table 2. A multiple comparison analysis was carried out to assess significant differences among the samples. Fisher's least significant differences (LSD) test was used to describe means with 95% confidence. Data on instrumental texture parameters during storage were evaluated by repeated measures analysis of variance (ANOVA). The results obtained allowed establishing staling behaviour of enzyme-supplemented bread crumb. Statgraphics Plus V5.1 and Statistica V6 programs were used as statistical analysis software.

Results and discussion

Dynamic viscoelastic properties of enzyme-supplemented doughs.

Individual effects of enzymes on dynamic moduli of doughs are showed in Table 3. Except GO and LAC, all enzymes affected significantly ($p<0.05$) the rheological behaviour of dough. TG and PROT modified dough rheology at all studied resting periods. However AMYL and XYL only had a significant effect on mentioned moduli after 180 min of incubation. The addition of TG led to a significant increase in elastic (G´), viscous (G˝) and complex (G*) moduli of doughs. These results were similar to those obtained by previous investigations (Caballero et al., 2005; Gujral & Rosell, 2004b; Köksel, Sivri, Ng, & Steffe, 2001; Larre et al., 2000) and confirmed the strengthening action exerted by TG due to its cross-linking effect on different flour protein fractions (Autio et al., 2005; Bauer et al., 2003a; Gerrard et al., 2001; Larre et al., 2000; Rosell et al., 2003). All dynamic moduli showed an steady increase with increasing incubation time, which proved the cumulative effect of TG. PROT diminished significantly elastic (G´) and complex (G*) moduli, whereas decrease in viscous modulus (G˝) was only significant ($p<0.05$) after a 180 min resting period. The weakening effect of PROT was also related with the decrease in resistance to extension observed by Indrani, Prabhasankar, Rajiv, & Venkateswara-Rao (2003). Proteinase activity affects specially to glutenins (Bombara, Anon, & Pilosof, 1997), which would alter the elasticity of the gluten complex.

Both polysaccharide-degrading enzymes promoted a similar significant decrease of all dynamic moduli analysed when samples were incubated during 180 min. Martínez-Anaya & Jiménez (1997; 1998) stated that hydrolytic

enzymes acting on carbohydrates induce a quick response in dough rheology and their action continue during resting.

Analysis of second order interactive effects of design factors (enzymes) on viscoelastic properties of dough revealed significant ($p<0.05$) interactions between TG and XYL, and between AMYL and PROT (data not shown). The protein polymerisation promoted by TG counteracted the softening effect of XYL after a large resting period. These results were consistent with those obtained after individual addition of both enzymes but disagreed with the synergistic diminution of uni- and bi-axial extensibility by the combination of TG and XYL observed by Collar & Bollaín (2004).

Bread quality of enzyme-supplemented doughs.

Bread quality parameters of doughs were significantly ($p<0.05$) affected by individual enzyme addition, except when LAC was used (Table 4). The greater effect was induced by TG, since this enzyme widely modified morphometric, textural and crumb grain properties of fresh pan breads. TG decreased significantly loaf specific volume but did not produce changes in its shape. The strengthening effect and dough extensibility reduction promoted by TG, probably decreased dough extension during fermentation and oven-spring. According to previous findings, the loaf volume could be only increased when additional water was applied (Autio et al., 2005), and when a poor baking quality flour was used together with TG (Basman, Köksel, & Perry, 2002). Single presence of TG led to a significant increase of hardness, cohesiveness, gumminess, chewiness and resilience of bread crumb. Crumb grain profile of TG-supplemented breads showed brighter crumb, smaller cells, greater cell

density and grain uniformity, and smaller void fraction and cell wall thickness. These results denote a finer and more uniform overall structure, which is consistent with an improved bread crumb grain (Sapirstein, 1999). Similar textural and crumb grain profiles have been stated previously by means of sensorial and instrumental studies of breads prepared with TG (Basman et al., 2002; Bauer, Koehler, Wieser, & Schieberle, 2003b; Collar & Bollaín, 2005a; Collar, Bollaín, & Angioloni, 2005; Gerrard, Fayle, Wilson, Newberry, Ross, & Kavale, 1998).

GO-supplemented doughs yielded loaves with an increased height/width ratio, characterised by more elastic and cohesive crumbs. Polysaccharide-degrading enzymes and PROT exercised similar suitable effects on pan bread quality parameters. Their use led to better shape, greater specific volume and void fraction of loaves. This behaviour was more marked when PROT was added to dough, and came accompanied by significant decreases in crumb hardness, gumminess and chewiness. Additionally, PROT gave more elastic crumb and a coarser bread crumb structure, which was characterized by greater cells, less cell density and fewer grain uniformity. Moreover, AMYL also increased mean cell area and decreased crumb elasticity. A more open gluten network formed by fibrous elements has been suggested by Blaszczak, Sadowska, Rosell & Fornal (2004) as the responsible for the higher elasticity and lower hardness of the crumb after treatments with AMYL.

Analysis of second order interactive effects of design factors on bread quality parameters revealed significant ($p<0.05$) interactions between TG and all the other enzymes except GO (Tables 5 and 6). LAC addition to TG containing

doughs only modified significantly crumb grain features, yielding loaves with less crumb brightness and cell density, but greater mean cell area and cell wall thickness than those obtained by the treatment with singly TG. Through simultaneous arabinoxylans gelation (Figueroa-Espinoza & Rouau, 1998) and oxidative action (Labat, Morel, & Rouau, 2000), LAC promoted a finer crumb structure than control samples. However, this enzyme would favour the interference of pentosans in glutenins aggregation (Primo-Martín et al., 2003), modifying TG strengthening effect and resulting in a coarser crumb. Moreover, AMYL, XYL and PROT exerted a softener effect on the crumb of TG-supplemented pan breads, leading to significant decreases in hardness, gumminess and chewiness of samples. Interactive effect of TG and XYL on bread quality could arise from rheological changes, which were consistent, in turn, with the release of pentosans from gluten network (Primo-Martín et al., 2003).

TG and PROT showed a significant synergistic effect on height/width ratio and specific volume of loaves. Likewise, PROT gave a more marked diminution of hardness and related parameters than AMYL or XYL, exhibiting values even lower than control samples. Crumb grain profile was also significantly affected by TG/PROT interaction. PROT addition increased void fraction and decreased grain uniformity of TG-treated samples. These results denoted that the hydrolytic effect of PROT, probably counteracted excessive protein polymerisation catalyzed by TG, making possible a better dough development during fermentation and oven-spring. Gottmann & Sproessler (1994) proved an undesired loss of extensibility after TG addition, and proposed its combination with a protease in order to avoid it.

AMYL and PROT combination led to significant improvement of loaf shape, although increase in height/width ratio was the same to that individually promoted by AMYL. Similar behaviour was observed in crumb void fraction, which value was also substantially higher than the one obtained for control samples. However, hardness, gumminess and chewiness clearly showed another trend, suggesting a significant synergistic effect of AMYL and PROT combination. GO and PROT combined synergistically improved loaf height/width ratio and loaf specific volume. The enhancement of this parameter was comparable with that obtained for singly PROT treatment.

LAC interacted significantly with PROT and XYL, to produce changes that essentially affected to the crumb grain pattern of loaves. LAC promoted a finer crumb grain, whereas PROT addition gave greater cells. However, the combined use of these enzymes led to a coarser structure, denoting a protein weakening effect. The interference of pentosans in the aggregation of gluten due to LAC action (Primo-Martín et al., 2003), would prevail over disulfide linkages promotion, inducing, in the presence of PROT, gas cells coalescence phenomena. Simultaneous supplementation with LAC and XYL gave rise to significant effects on crumb brightness, cell density and cell wall thickness.

Enzyme-supplemented bread staling during storage.

Repeated measures analysis of variance enabled us to establish the single and the second-order interactive effects of the enzymes on the trend and extent of variation of instrumental texture parameters of enzyme-supplemented pan breads during the storage. Significant effects ($p<0.05$) were provided by TG, AMYL, XYL and PROT when they were used individually. TG significantly

affected to the evolution of all textural parameters in the time. Bread staling increased by TG addition, and affected specially to hardness (Figure 1a), chewiness and gumminess. These results differed from those obtained with enriched formulation (Collar & Bollaín, 2005a). Martin, Zeleznak, & Hoseney, (1991) suggested that interactions between the swollen starch granules and the protein network actively contribute to crumb firming. Through microscopic analysis of bread crumb, significant differences in starch-protein matrix have been detected in the course of storage (Blaszczak et al., 2004). TG-induced strengthening effect could increase such interactions and favour bread staling and simultaneous crumb elasticity preservation during storage. The affinity to water promoted by TG in gluten (Gerrard et al., 1998) could also limit the water availability for starch and accelerate its retrogradation.

On the contrary, AMYL, XYL and PROT exhibited a significant antistaling effect (Figures 1b, 1c and 1d). PROT showed the most marked effect on reducing hardness, which came accompanied by a significant slowing down in gumminess and chewiness evolution in the time (data not shown).

According with the conclusions of Armero & Collar (1998), crumb firming during storage mainly depends on initial crumb firmness. Therefore, softener effect of AMYL, XYL and PROT (Figure 1) would justify partially its influence on firming kinetics. Alpha-amylase has been proved to be useful for reducing amylopectin retrogradation and the firming rate of wheat bread crumb (Champenois, della Valle, Planchot, Buleon, & Colonna, 1999) and rice bread crumb (Gujral, Haros, & Rosell, 2003). Although Sahlström & Brathen (1997) indicated that the mechanisms governing crumb firmness and the retrogradation of amylopectin

seemed to be different, Morgan, Gerrard, Every, Ross, & Gilpin (1997) suggested that starch retrogradation is sufficient to cause bread firming. Through studies carried out on model systems, Rojas, Rosell, & Benedito de Barber (2001) concluded that maltodextrins were responsible for the anti-staling effect promoted by addition of α-amylase to bread formulation. They proposed the existence of a mechanism of partial obstruction of starch retrogradation. Jiménez & Martínez-Anaya (2001) proved that water-insoluble pentosans (WIP) were positively correlated with crumb elasticity and hardness during storage. XYL would lead to cleavage of the backbone of arabinoxylans, with the consequent release of water and WIP diminution (Rouau, El Hayek, & Moreau, 1994), which could explain the positive effects of XYL in bread freshness. Similarly, the improvement of bread shelf-life through PROT addition possibly would be tied with the increase of the water available for starch, in conjunction with a simultaneous diminution of starch-protein interactions as consequence of the hydrolysis of peptide bonds in the protein molecules. Babiker, Fujisawa, Matsudomi, & Kato (1996) previously reported an increase in the hydrophobicity of protease-treated gluten.

Statistical analysis of the textural data during storage proved significant ($p<0.05$) second-order interactive effects between enzymes. AMYL, XYL and PROT diminished significantly the staling effect promoted by TG. Their action was showed clearly through crumb hardness evolution (Figures 2a, 2b and 2c). However, the behaviour of these samples did no reach to that of single AMYL, XYL and PROT-supplemented breads. The mechanisms by which these enzymes slowed down staling kinetics of TG-treated samples probably were rather different. Whilst XYL and AMYL would act on dough polysaccharide

fraction, PROT directly would counteract TG-action, by simultaneously acting on dough protein fraction. Besides their ability to modify the degree of protein polymerisation and consequently, the starch-protein interactions, TG/PROT combination has been reported as responsible for changing the number of exposed hydrophobic residues (Babiker et al.,1996), which could alter dough water availability. Using dynamic and static deformation measurements, Bollaín, Angioloni, & Collar (2005) confirmed synergistic interactions regarding staling behaviour of breads formulated with TG/XYL and TG/AMYL combinations. Addition of bacterial alpha-amylase to TG-supplemented proved to significantly slow down the staling kinetics determined as cohesiveness and resilience (Collar & Bollaín, 2005a).

AMYL and PROT also combined synergistically to decrease bread staling during storage, as could be deduced from their significant effect on crumb firming kinetics (Figure 2d).

Conclusions

Among all gluten cross-linking enzymes analysed, dynamic rheological test only showed a significant single effect of transglutaminase. Protease decreased dynamic moduli at all studied resting periods, whilst polysaccharide-degrading enzymes modified dough rheology after 180 min of incubation. Statistical analysis of viscoelastic properties revealed that simultaneous use of TG and XYL could be an interesting alternative for avoiding excessive dough strengthening promoted by TG.

Bread quality parameters of doughs were significantly affected by individual enzyme addition, except when LAC was used. The greater effect was provided by TG, since this enzyme widely modified morphometric, textural and crumb grain properties of fresh pan breads. Polysaccharide-degrading enzymes and PROT led to better shape, greater specific volume and void fraction of loaves. Except GO, all enzymes showed significant interactive effects with TG. In accordance with crumb hardness evolution, it was proved that AMYL, XYL and PROT were able to diminish the staling effect promoted by TG. AMYL and PROT also combined synergistically to decrease bread firming during storage. Therefore, the antistaling effect of PROT was confirmed. Likewise, results suggest that, through different mechanisms, dough protein and polysaccharide fractions actively contribute to bread staling kinetics.

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2

Table 1. Quality attributes of wheat flour

	Flour
Chemical composition	
Protein (% d. wt.)	11.00
Ash (% d. wt.)	0.58
Moisture) (% d. wt.)	12.16
Consistogram	
Water absorption (%)	52.8
Alveogram	
Deformation energy (10^{-4} J)	146
Curve configuration ratio	0.35
Gluten Index	
Gluten Index (%)	94
Dry Gluten (%)	9.00
Wet Gluten (%)	26.60
Falling Number	
Time (s)	405

d. wt. : dry weight

2

Table 2. Half fraction factorial design 2^6 for sampling

Sample no.	Factors [a]					
	A	B	C	D	E	F
1	0	0	0	0	0	0
2	0	1	1	0	1	1
3	0	1	0	0	1	0
4	0	1	0	1	1	1
5	0	1	1	1	1	0
6	0	0	0	1	1	0
7	0	0	1	1	1	1
8	1	1	1	1	0	0
9	1	0	0	1	1	1
10	1	1	0	1	0	1
11	0	1	1	0	0	0
12	0	1	0	1	0	0
13	1	1	1	1	1	1
14	1	0	0	0	0	1
15	0	1	0	0	0	1
16	0	0	1	1	0	0
17	1	0	1	1	1	0
18	0	0	0	1	0	1
19	1	0	0	1	0	0
20	1	0	1	0	0	0
21	1	0	1	0	1	1
22	1	1	0	1	1	0
23	1	1	0	0	0	0
24	1	1	1	0	1	0
25	1	1	0	0	1	1
26	1	1	1	0	0	1
27	0	0	0	0	1	1
28	0	0	1	0	1	0
29	1	0	0	0	1	0
30	0	0	1	0	0	1
31	0	1	1	1	0	1
32	1	0	1	1	0	1

4 [a]Levels (0,1) of factors (A to F): A = Transglutaminase (TG): none (0), 500 mg/100g flour (1); B = Glucose oxidase (GO): none (0), 3 mg/100 g flour (1); C = Laccase (LAC): none (0), 20 μl/100 g flour (1); D =
6 Amilase (AMYL): none (0), 1 mg/100 g flour (1); E = Pentosanase (XYL): none (0), 6 mg/100 g flour (1); F=Protease (PROT): none (0), 20 μl/100 g flour (1).

Table 3.- Single effects of design factors on viscoelastic properties of enzyme-supplemented doughs.

Parameter	Units	Overall mean	TG[a]			GO		LAC		AMYL			XYL			PROT		
			0	1		0	1	0	1	0	1		0	1		0	1	
G'_{30min}	Pa	10354	**8722**	**11985**	*	10090	10618	10750	9958	10903	9805		10803	9905		**10987**	**9720**	*
G''_{30min}	Pa	3523	**3241**	**3806**	*	3463	3584	3642	3405	3654	3393		3607	3440		3695	3352	
G^*_{30min}	Pa	10940	**9301**	**12579**	*	10671	11210	11353	10528	11491	10389		11398	10483		**11603**	**10278**	*
G'_{60min}	Pa	11115	**8466**	**13765**	*	10991	11239	11603	10628	11996	10234		11748	10483		**12032**	**10199**	*
G''_{60min}	Pa	3607	**3167**	**4048**	*	3588	3626	3740	3474	3811	3403		3740	3474		3850	3364	
G^*_{60min}	Pa	11700	**9036**	**14364**	*	11577	11823	12196	11204	12608	10793		12344	11056		**12644**	**10756**	*
G'_{180min}	Pa	12950	**7824**	**18075**	*	12555	13344	13523	12376	**14637**	**11263**	*	**14471**	**11429**	*	**14800**	**11099**	*
G''_{180min}	Pa	3890	**3018**	**4763**	*	3771	4009	4036	3745	**4281**	**3499**	*	**4231**	**3549**	*	**4322**	**3459**	*
G^*_{180min}	Pa	13540	**8393**	**18688**	*	13123	13958	14138	12943	**15277**	**11804**	*	**15097**	**11984**	*	**15444**	**11636**	*

[a]See table 2 for levels of design factors.
* The effect of the factor is significant with a significance level of 95% ($p<0.05$)

Table 4.- Single effects of design factors on bread quality of enzyme-supplemented doughs.

Parameter	Units	Overall mean	TG[a]			GO			LAC			AMYL			XYL			PROT		
			0	1		0	1		0	1		0	1		0	1		0	1	
Height/Width ratio		0.87	0.87	0.86		**0.84**	**0.90**	*	0.86	0.87		**0.84**	**0.89**	*	**0.84**	**0.89**	*	**0.81**	**0.92**	*
Specific volume	cm^3/g	3.73	**3.85**	**3.61**	*	3.67	3.80		3.73	3.74		**3.56**	**3.91**	*	**3.53**	**3.94**	*	**3.40**	**4.01**	*
Hardness	gf	376	**297**	**456**	*	402	351		375	378		**451**	**301**	*	**443**	**310**	*	**494**	**259**	*
Cohesiveness		0.8293	**0.8176**	**0.8409**	*	**0.8217**	**0.8368**	*	0.8276	0.8309		0.8290	0.8295		0.8303	0.8282		0.8257	0.8328	
Gumminess	gf	312	**242**	**382**	*	330	293		310	313		**374**	**250**	*	**367**	**256**	*	**408**	**216**	*
Chewiness	gf	306	**237**	**374**	*	323	288		304	307		**366**	**245**	*	**359**	**252**	*	**398**	**213**	*
Springiness		0.9823	0.9820	0.9826		0.9809	0.9837		0.9823	0.9822		0.9821	0.9824		0.9800	0.9846		**0.9792**	**0.9853**	*
Resilience		0.4516	**0.4446**	**0.4586**	*	**0.4437**	**0.4595**	*	0.4515	0.4518		**0.4606**	**0.4426**	*	0.4569	0.4463		0.4523	0.4509	
Crumb brightness		160	**151**	**169**	*	160	160		159	161		159	161		159	162		163	158	
Mean cell area	mm^2	1.48	**1.78**	**1.18**	*	1.49	1.46		1.50	1.45		**1.41**	**1.54**	*	1.44	1.52		**1.33**	**1.63**	*
Cell density	$cells/cm^2$	30	**23**	**37**	*	30	31		31	30		31	30		30	31		**34**	**27**	*
Void fraction	%	41.5	**42.8**	**40.2**	*	41.0	40.2		41.4	41.6		**40.5**	**42.5**	*	**40.7**	**42.3**	*	**40.1**	**42.9**	*
Cell wall thickness	mm	0.75	**0.81**	**0.69**	*	0.76	0.73		0.76	0.74		0.76	0.74		0.77	0.73		0.73	0.77	
Grain uniformity		11.7	**7.2**	**16.1**	*	11.7	11.7		11.9	11.4		12.6	10.7		12.3	11.1		**14.5**	**8.8**	*

[a]See table 2 for levels of design factors.

* The effect of the factor is significant with a significance level of 95% ($p<0.05$)

Table 5.- Second-order interactive effects of design factors on morphometric and textural properties of enzyme-supplemented fresh pan breads

Parameter	Units	Overall mean	Level[a]	TG/ GO	TG/ LAC	TG/ AMYL	TG/ XYL	TG/ PROT	GO/ LAC	GO/ AMYL	GO/ XYL	GO/ PROT	LAC/ AMYL	LAC/ XYL	LAC/ PROT	AMYL/ XYL	AMYL/ PROT	XYL/ PROT
Height/Width ratio		0.87	00					**0.86***				**0.77***					**0.76***	
			01					**0.88**				**0.91**					**0.92**	
			10					**0.77**				**0.86**					**0.86**	
			11					**0.96**				**0.93**					**0.92**	
Specific volume	cm^3/g	3.73	00					**3.73***				**3.22***						
			01					**3.97**				**4.11**						
			10					**3.06**				**3.57**						
			11					**4.17**				**4.02**						
Hardness	gf	376	00			**327***	**318***	**362***									**625***	
			01			**266**	**275**	**231**									**277**	
			10			**576**	**568**	**625**									**362**	
			11			**337**	**345**	**287**									**240**	
Cohesiveness		0.8293	00															
			01															
			10															
			11															
Gumminess	gf	312	00			**266***	**260***	**294***									**516***	
			01			**217**	**224**	**190**									**231**	
			10			**481**	**475**	**522**									**300**	
			11			**283**	**289**	**242**									**200**	
Chewiness	gf	306	00			**262***	**254***	**288**									**503**	**482**
			01			**212**	**220**	**187**									**228**	**236**
			10			**470**	**464**	**509**									**293**	**314**
			11			**278**	**284**	**239**									**198**	**190**
Springiness		0.9823	00															
			01															
			10															
			11															
Resilience			00															
			01															
			10															
			11															

[a]See table 2 for levels of design factors.

* The effect of the factor is significant with a significance level of 95% ($p<0.05$)

Table 6.- Second-order interactive effects of design factors on crumb grain characteristics of enzyme-supplemented fresh pan breads

Parameter	Units	Overall mean	Level[a]	TG/ GO	TG/ LAC	TG/ AMYL	TG/ XYL	TG/ PROT	GO/ LAC	GO/ AMYL	GO/ XYL	GO/ PROT	LAC/ AMYL	LAC/ XYL	LAC/ PROT	AMYL/ XYL	AMYL/ PROT	XYL/ PROT
Crumb brightness			00		**147***									**155***				
			01		**156**									**163**				
			10		**172**									**163**				
			11		**167**									**160**				
Mean cell area	mm^2		00		**1.91***										**1.42***			
			01		**1.64**										**1.58**			
			10		**1.09**										**1.23**			
			11		**1.26**										**1.68**			
Cell density	$cells/cm^2$		00		**20***									**28***				
			01		**26**									**34**				
			10		**41**									**32**				
			11		**34**									**28**				
Void fraction	%		00					**42.4***									**37.9***	
			01					**43.1**									**43.0**	
			10					**37.7**									**42.2**	
			11					**42.7**									**42.8**	
Cell wall thickness	mm		00		**0.87***									**0.82***				
			01		**0.75**									**0.70**				
			10		**0.65**									**0.72**				
			11		**0.73**									**0.75**				
Grain uniformity			00					**8.3***										
			01					**6.2**										
			10					**20.7**										
			11					**11.4**										

[a]See table 2 for levels of design factors.

* The effect of the factor is significant with a significance level of 95% ($p<0.05$)

Table 7.- Single and second-order interactive effects of design factors on staling kinetics parameters during storage of enzyme-supplemented pan breads

Design factor	TEXTURAL PARAMETERS					
	Hardness (gf)	Cohesiveness	Gumminess (gf)	Chewiness (gf)	Springiness	Resilience
TG	✳	✳	✳	✳	✳	✳
GO						
LAC						
AMYL	✳	✳			✳	✳
XYL	✳	✳	✳	✳	✳	
PROT	✳		✳	✳		
TG/GO		✳				✳
TG/LAC						
TG/AMYL	✳					
TG/XYL	✳			✳		
TG/PROT	✳		✳	✳		
GO/LAC						
GO/AMYL						
GO/XYL						
GO/PROT						
LAC/AMYL						
LAC/XYL						
LAC/PROT						
AMYL/XYL		✳				
AMYL/PROT	✳					
XYL/PROT		✳			✳	✳

✳ The effect of the factor is significant with a significance level of 95% ($p<0.05$)

FIGURE CAPTIONS

Figure 1: Significant single effects of design factors on crumb hardness evolution during storage of enzyme-supplemented pan breads [TG (a), AMYL (b), XYL (c) and PROT (d)]. Bars describe the standard deviation. Continuous line represents the evolution of bread crumb hardness in presence of the factor, whilst discontinuous line represents the evolution of bread crumb hardness in absence of the factor. (See table 2 for codes of design factors) [Significance level of 95% ($p<0.05$)].

Figure 2: Significant second-order interactive effects of design factors on crumb hardness evolution during storage of enzyme-supplemented pan breads [TG/AMYL (a), TG/XYL (b), TG/PROT (c) and AMYL/PROT (d)]. Bars describe the standard deviation. (See table 2 for codes of design factors) [Significance level of 95% ($p<0.05$)].

Fig. 1

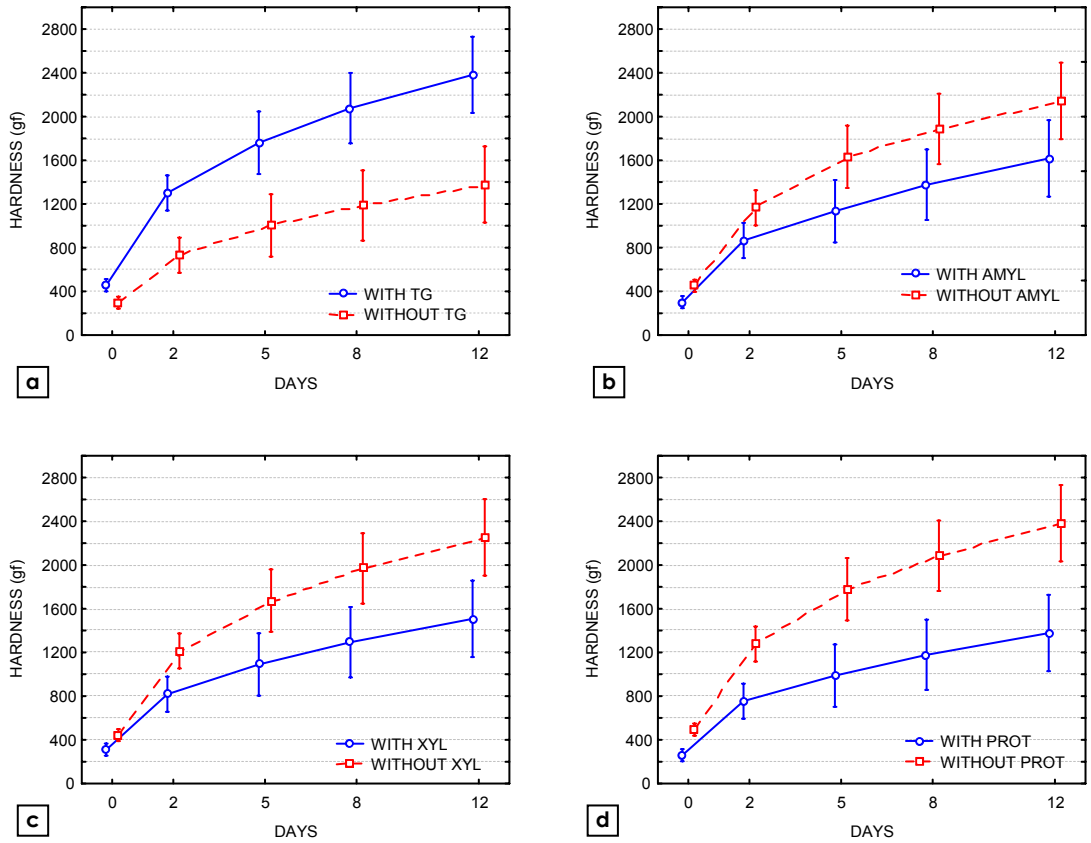
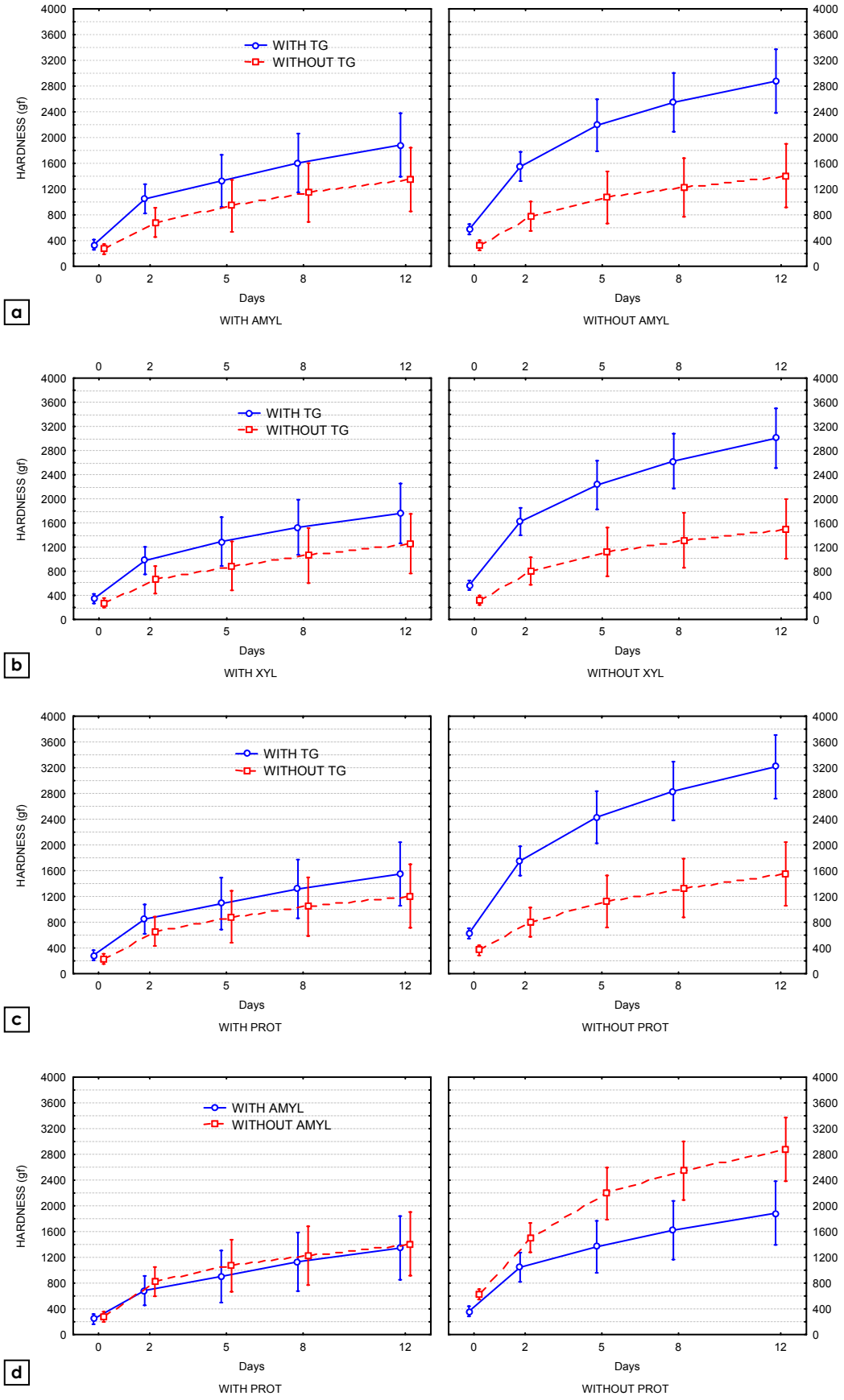

HARDNESS (gf)
DAYS
WITH TG
WITHOUT TG
a
WITH AMYL
WITHOUT AMYL
b
WITH XYL
WITHOUT XYL
c
WITH PROT
WITHOUT PROT
d

Fig. 2


a
HARDNESS (gf)
WITH TG
WITHOUT TG
Days
WITH AMYL
WITHOUT AMYL
b
WITH XYL
WITHOUT XYL
c
WITH PROT
WITHOUT PROT
d
WITH AMYL
WITHOUT AMYL
WITH PROT
WITHOUT PROT