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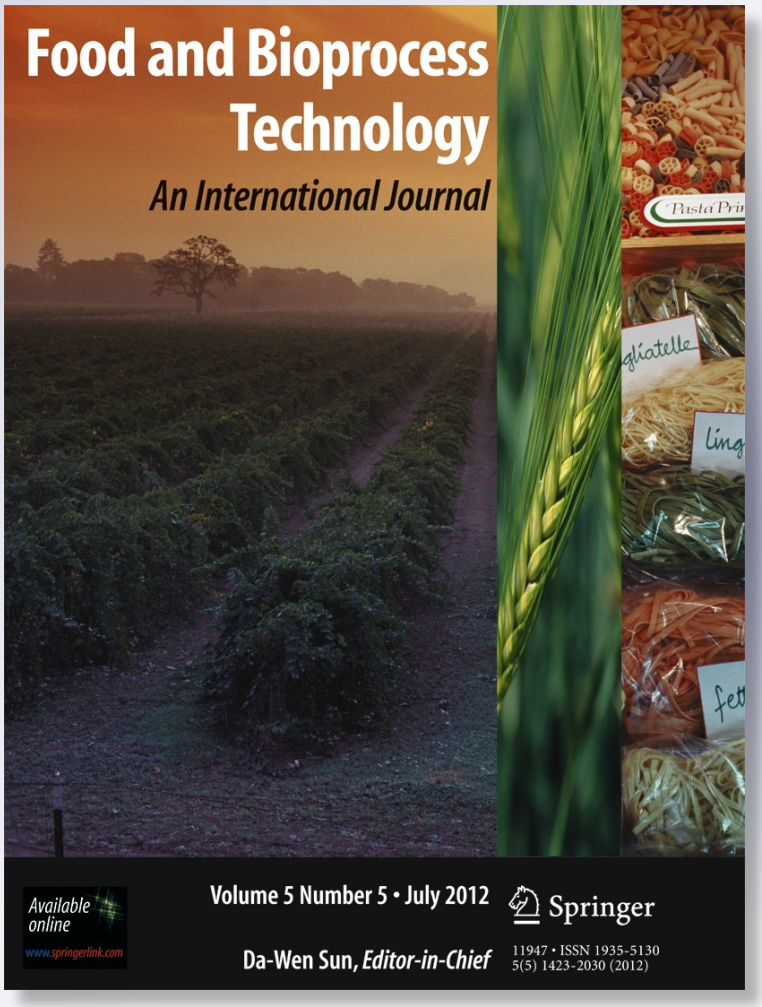

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ORIGINAL PAPER

Use of Enzymes to Minimize Dough Freezing Damage

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Abstract The purpose of this investigation was to study the effect of pentosanase (Pn), glucose oxidase (Gox), and transglutaminase (TG) on frozen dough (−18 °C) and their influence on minimizing the damage caused by frozen storage. Bread characteristics were analyzed on day 0; after 3 and 9 weeks of frozen storage, specific loaf volume, crust color, and crumb texture and structure were analyzed. Dough expansion capacity and dough stickiness, extensibility, and viscoelasticity were determined. Frozen dough with high levels of Gox developed a larger bread volume than control dough (without added enzyme). The damage percentage caused by frozen storage in Gox samples was lower than in control samples, indicating that Gox increased dough strength and counteracted the depolymerization effect of gluten produced by ice crystal formation and the release of reducing substances from dead yeast cells during freezing. Samples with Pn developed a large bread volume after 9 weeks of frozen storage because of the formation of smaller pentosans, which result from Pn enzyme action. These pentosans were located in protein–starch–CO_2 matrix interfaces and increased dough expansion capacity without gas loss, thus allowing a higher expansion during proofing. The intermediate level of TG was the only one to present a larger bread volume from frozen dough than control. The new isopeptidic bonds introduced by TG in the gluten proteins helped to mitigate the damage caused by dough freezing.

Keywords Frozen dough · Transglutaminase · Pentosanase · Glucose oxidase

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Introduction

Fresh breads have a short shelf-life because physical and chemical changes known as "bread staling" take place during storage. Dough freezing technology is largely used to reduce the economic losses resulting from bread staling, offering a permanently fresh product to the consumers (Matuda et al. 2005). However, dough frozen storage for long periods decreases the quality of the end product (Inoue and Bushuk 1992; Ribotta et al. 2001). In the frozen dough methodology, the dough is developed and then bread pieces are formed and frozen until the center itself reaches −18 °C. At the points of sale, frozen dough pieces are thawed, proofed, and baked as the products are consumed (Giannou et al. 2003).

Frozen dough loss of baking quality has been ascribed to dough weakening and to a reduction in both yeast viability and activity. Gradual loss of dough strength during frozen storage has been attributed to the reduction in gluten cross-linking caused by ice crystallization, the release of reducing substances from yeast, and the water redistribution provoked by a modification in the water binding capacity of dough constituents (Varriano-Marston et al. 1980; Inoue and Bushuk 1992; Autio and Sinda 1992; Ribotta et al. 2001, 2003). These phenomena may produce a loss in the gas retention capacity during fermentation, reflected by

lower bread volume and an increase in fermentation time (Ribotta et al. 2003). Freezing reduces yeast activity considerably. Several authors showed that fast freezing reduces the CO_2 production capacity and the number of viable yeast (Wolt and D'Appolonia 1984; Autio and Sinda 1992; Inoue et al. 1994; El-Hady et al. 1996). Even when several explanations have been posed to account for the weakening behavior of the dough, it is very likely that this behavior takes place because of a combination of all of them. This problem could be solved by the use of additives to mitigate the damage caused by freezing.

Pentosanase (Pn) or microbial endoxylanase (EC 3.2.1.8) catalyzes the hydrolysis of arabinoxylans (pentosans) in wheat flour (Courtin and Delcour 2001). When the enzyme exerts its effect on water-insoluble arabinoxylans, these polymers decrease in the size and molecular weight and become water-soluble components. Water-soluble arabinoxylans have a positive effect on dough and bread properties; they stabilize gas cell, they increase the viscosity of the dough aqueous phase, they increase dough stability, and they even improve bread characteristics, such as bread specific volume, firmness, and crumb structure (Gan et al. 1995; Steffolani et al. 2010). On the other hand, the endoxylanase that affects water-soluble arabinoxylans mainly reducing its molecular weight has less or no effect on bread quality (Courtin et al. 1999; Courtin and Delcour 2001).

The glucose oxidase (Gox) enzyme (EC 1.1.3.4), in the presence of oxygen, catalyzes the oxidation of α-D-glucose to α-D-gluconolactone and H_2O_2. The H_2O_2 oxidizes thiol groups of gluten proteins to form disulfide bonds (Haarasilta and Pullinen 1992). Several research groups observed an increase in dough tenacity and elasticity, and a decrease in dough extensibility upon Gox addition (Rosell et al. 2003; Bonet et al. 2006; Davidou et al. 2008; Steffolani et al. 2010). Other researchers observed that it had no effect or a negative one on loaf volume and that it improved crumb texture and strength (Rasiah et al. 2005; Steffolani et al. 2010).

The transglutaminase (TG) enzyme (EC 2.3.2.13) catalyzes the formation of intra- or intermolecular e-(g-glutamyl) lysine isopeptide bonds. The addition of TG to wheat flour during bread-making alters dough characteristics. TG improves dough elasticity and bread crumb strength (Gerrard et al. 1998) because it transforms weak gluten into strong gluten, affecting dough rheological behavior (Larré et al. 2000). However, it has also been reported that TG decreases dough extensibility (Rosell et al. 2003), which may lead to an undesirable reduction in loaf volume when it becomes too strong. High levels of TG result in low-volume breads because dough resistance is higher and CO_2 pressure is not enough to achieve dough expansion (Steffolani et al. 2010). Huang et al. (2008) observed that TG improved frozen dough bread quality; the enzyme allowed us to obtain breads from frozen dough—which had been stored for 5 weeks—with high specific volume and low crumb firmness.

Although the effect of these enzymes on the improvement of bread quality has been studied by several authors, some aspects of their mechanism of action during dough freeze storage have not been completely elucidated until now. Therefore, the aim of this work was to study the effect of Pn, Gox, and TG on frozen dough and the influence of these enzymes on the minimization of the damage caused by frozen storage.

Materials and Methods

Materials

Commercial wheat flour utilized in all experiments was provided by a local milling company (Molinos Campodónico Ltda., La Plata, Argentina). Wheat flour parameters were: deformation energy (W)=323×10^{-4} J, tenacity (P)=101 mm, extensibility (L)=89 mm, P/L=1.13 (AACC International 2000), moisture=13.1±0.2%, and protein=10.9±0.3% (Kjeldahl method, $N\times5.7$). Enzymes were included; glucose oxidase (Gox, gluzyme mono BG from Novozyme, Denmark) 100,000 U/g activity (1 unit of Gox is defined as the amount of enzyme that oxidizes 1 μmol of *o*-dianisidine/min at 25 °C), active microbial transglutaminase wm (TG, from Apliena Ajinomoto, Japan), 100 U/g activity (1 unit of TG is defined as the amount of enzyme that releases 1 μmol of hydroxamic acid/min at 37 °C), endo-β-1,4-xylanase (Pn, pentopan mono BG from Novozyme, Denmark) 2,500 FXU/g activity (fungal xylanase units/g). The enzyme concentrations (grams enzyme product per 100 grams flour) utilized were: Gox=0.001%, 0.005%, and 0.01%; TG=0.01%, 0.1%, and 0.5%; Pn=0.006%, 0.012% and 0.018%. The concentrations of Gox and Pn used were recommended by the manufacturers, and TG concentrations were selected according to previous publications (Basman et al. 2002; Collar et al. 2005; Caballero et al. 2007; Ribotta et al. 2010; Roccia et al. 2010). Wheat flour without enzyme was used as control. All the reagent chemicals used were of analytical grade.

Bread-Making Procedure

Basic bread dough formula on 100-g flour basis consisted of 3% (*w*/*w*) compressed yeast, 2.2% (*w*/*w*) salt, 58.5% water (optimum level), and enzymes. Yeast and salt were first dissolved in water separately, while the remaining ingredients were added in their solid state. Ingredients were mixed for 9 min in an Argental L-20 mixer (Argentina). The resulting dough was allowed to rest for 15 min at 30 °C. The dough was divided into 120-g pieces, hand-molded, and

allowed to rest for 15 min at 30 °C. Then, dough pieces were molded into a loaf shape (Braesi MB 350, Brazil) and separated into two batches. For one batch, dough pieces were proofed at 30 °C (96% relative moisture, rm) up to its maximum volume increment and baked at 210 °C for 18 min (unfrozen dough) in a rotational gas oven (Ciclo Ingeniería, Argentina). For the other batch, dough pieces were placed on individual trays and wrapped up in polyethylene bags immediately after shaping. Then, these dough pieces were frozen and stored at −18 °C in a freezer (Frare, Argentina) for 3 and 9 weeks. After frozen storage, the samples were thawed for 1 h at 30 °C (70% rm), proofed at 30 °C (96% rm) up to optimum development, and baked as described above. Two hours after baking, the loaves were weighed, and bread loaf volume was determined by rapeseed displacement. The bread specific volume (BSV) was expressed as the volume/weight of finished bread. Each baking was conducted in duplicates.

Bread Properties

Crust Color Bread crust color was determined with a Minolta 508d spectrophotometer, 8-mm measurement aperture, D65 illuminant, 10°-angle of observer, according to Approved Methods 14–22 (AACC International 2000). Crust color was measured on the top of each bread loaf. At least four readings were taken from each bread loaf, and four bread loaves were analyzed in each assay; readings were recorded as CIE-LAB, L^* (lightness), a^* (red-green), and b^* (yellow-blue) values.

Crumb Texture Texture profile analysis was performed by using a TA.XT2i Texture Analyser (Stable Micro Systems, Surrey, UK) equipped with a 25-kg load cell. A perspex cylinder probe of 2.5 cm diameter was attached to a moving crosshead. Two hours after baking, the bread loaves were cut, and two slices (2.5 cm thick) were subjected to a double compression–decompression cycle under the following conditions with 1 mm/s crosshead speed and 40% maximum deformation. The texture profile parameters of crumb firmness, and chewiness were determined using the Texture Expert 1.22 software (Stable Micro Systems, Surrey, UK). Two slices of bread from each loaf were analyzed, and four bread pieces from each test point were used.

Crumb Structure For each bread loaf, two slices obtained from the central region were photographed with a digital camera (Canon, Mississauga, Canada; 2,048–1,536 image size). JPEG image file formats were analyzed with image analyzer software (Image J 1.38n; National Institute of Health, Bethesda, USA). Color images were converted to eight-bit 256 gray level images. Images were taken from the center (field of view (FOV)) of the slice. The segmentation method (conversion to a binary image) of the 256 gray level digital images was used to extract coherent information from raw image data. A threshold method was used for image segmentation. An algorithm (IsoData, software Image J 1.38n; National Institute of Health, Bethesda, USA) 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. A gray level histogram of each digital gray scale image was obtained by means of Image J software. A threshold level was obtained for each FOV and used to produce a binary image, where pixels with a gray level higher than the threshold value were associated to the air cells, while pixels with a gray level lower than the threshold value were associated to the wall cells. The evaluated crumb cell features were the total number of cells, the total cell area, the mean cell area, and the ratio between cell area and total area (CF). Grain uniformity was determined as the ratio of small to large cell counts (i.e., the ratio of the number of small cells to large ones, 4 mm^2); higher values indicate greater uniformity of crumb grain (Zghal et al. 2001).

Dough Properties

Dough Expansion Capacity Basic dough formula on a 100-g flour basis consisted of 3% (*w*/*w*) compressed yeast, 2.2% (*w*/*w*) salt, 58.5% water (optimum level), and enzymes. Ingredients were mixed in a Philips HR 1495 high-speed mixer with a dough hook for 2 min and allowed to rest for 5 min. Dough temperature was about 27 °C at this point. The resulting dough was allowed to rest for 15 min, and the bulk dough was subsequently sheeted by hand. The dough was then divided into 50-g pieces and rounded. For one batch, the dough was immediately wrapped up in polyethylene bags and frozen at −18 °C. After frozen storage, the samples were thawed for 45 min at 30 °C (70% rm). For the other batch, samples were immediately tested. To determine dough expansion capacity, two pieces (50-g) of dough (unfrozen and frozen/thawed) were immediately put into 500-ml calibrated graduated cylinders. Dough was pressed to achieve a smooth surface, and the cylinders were left for 150 min in a water bath at 30 °C. Dough rising was measured every 15 min (Ribotta et al. 2005). The proofing time and final volume were determined. Determinations were done in duplicate.

Dough Properties Bread dough was prepared following the dough formulation already indicated for the expansion assay, without yeast for the uniaxial extensibility and sticking tests and with yeast for the viscoelasticity test. Ingredients were mixed in a Philips HR 1495 mixer (Philips, Argentina) for 2 min and allowed to rest for 15 min in a fermentation cabinet at 30 °C and 70% rm. The

resulting dough was divided (30-g), hand-rounded, and wrapped up in thermally sealed polyethylene bags. Dough was frozen and stored at −18 °C. Frozen dough samples were thawed for 45 min in a cabinet at 30 °C. Unfrozen and thawed-frozen dough were tested. Different rheological properties, such as extensibility, stickiness, and viscoelasticity, were measured on dough pieces. All determinations were done at least in duplicate.

Dough Stickiness Dough textural properties were measured in triplicate with four trials per assay using a TA.XT2i Texture Analyzer (Stable Microsystems, Surrey, UK). Dough stickiness was determined using the SMS/Chen-Hoseney Dough Stickiness cell coupled with a 25-mm perspex cylinder probe (25-kg load cell). Dough stickiness or adhesiveness, measured by the force required to release the probe from the dough, was conducted using a force of 40-g through a distance of 4 mm at a pre-test speed of 0.5 mm/s, a test speed of 0.5 mm/s, and a post-test speed of 10 mm/s.

Dough Extensibility Measurements were performed with a TA.XT2i texture analyzer (Stable Microsystems, Surrey, UK) using the SMS/KIEFFER RIG for dough extensibility measurements, and unfrozen and thawed-frozen dough (20-g) was pressed to obtain the strip form and allowed to relax for 40 min. Ten strips by batch were placed on the platform, trimmed, and extended until their elasticity was exceeded and the dough broke up. The dough strips were extended at 3.3 mm/s (25-kg load cell). Maximum resistance to extension (Rm) and maximum extensibility (Em) were automatically calculated from the curves using the Texture Expert 1.22 (Stable Micro Systems, Surrey, UK).

Dough Viscoelasticity Tests were carried out in a rheometer (RS600, Haake, Karlsruhe, Germany) using a 1-mm gap plate–plate serrated geometry sensor (PP30). Unfrozen and thawed-frozen dough were placed on the lower plate, and excess dough protruding from the edge of the plate was carefully trimmed. Low-viscosity silicone was added around the plate edges to prevent dough dehydration. Before starting the oscillatory measurement, dough sample was held at rest for 2 min to allow the relaxation of any normal stress produced by sample loading. Temperature was kept constant (25 °C). Frequency sweeps at 0.03–100 Hz within the linear viscoelasticity range (strain= 0.016%) were performed. Storage modulus (G') was obtained at 1 Hz.

Statistical Analysis

INFOSTAT statistical software 2010p (Facultad de Ciencias Agropecuarias, UNC, Argentina) was used to perform the statistical analysis. A Fisher's test (LSD) was made in order to evaluate differences among samples, while the relationship between measured parameters was assessed by Pearson's test (significant level at $p \leq 0.05$).

Results and Discussion

Effect of Enzymes on Bread Quality

Bread specific volume from frozen dough is presented in Table 1. Significant decreases of bread specific volume were obtained from frozen dough after being stored for

Table 1 Effects of enzymes on specific volume of unfrozen and frozen bread

Sample	Unfrozen (cm^3/g)	Storage at −18 °C		
		Week 3 (cm^3/g)	Week 9 (cm^3/g)	Volume decrease by frozen storage (%)
Control	3.50 ± 0.06^{cde}	2.95 ± 0.02^{abc}	2.67 ± 0.01^{b}	23.74 ± 1.19^{d}
G1	3.64 ± 0.06^{e}	2.85 ± 0.01^{ab}	2.67 ± 0.00^{b}	26.64 ± 1.15^{d}
G2	3.38 ± 0.09^{bcd}	3.10 ± 0.08^{cd}	2.99 ± 0.11^{de}	11.57 ± 0.73^{b}
G3	3.19 ± 0.10^{b}	2.98 ± 0.04^{bc}	2.96 ± 0.05^{d}	7.30 ± 1.32^{a}
P1	4.10 ± 0.00^{f}	3.57 ± 0.15^{e}	3.00 ± 0.12^{de}	26.95 ± 2.93^{d}
P2	4.78 ± 0.17^{g}	3.60 ± 0.00^{e}	3.11 ± 0.02^{e}	35.01 ± 1.87^{e}
P3	4.70 ± 0.25^{g}	3.68 ± 0.04^{e}	3.11 ± 0.01^{e}	33.77 ± 3.34^{e}
T1	3.59 ± 0.02^{de}	3.03 ± 0.15^{bcd}	2.63 ± 0.01^{ab}	26.78 ± 0.23^{d}
T2	3.30 ± 0.04^{bc}	3.22 ± 0.23^{d}	2.81 ± 0.07^{c}	16.22 ± 0.01^{c}
T3	2.83 ± 0.05^{a}	2.72 ± 0.05^{a}	2.50 ± 0.05^{a}	11.66 ± 0.21^{b}

Different letters within a column indicate significantly different values ($p \leq 0.05$). G1, 0.001%; G2, 0.005%; and G3, 0.01% of glucose oxidase. T1, 0.01%; T2, 0.1%; and T3, 0.5% of transglutaminase. P1, 0.006%; P2, 0.012%; and P3, 0.018% of pentosanase. Unfrozen: bread obtained from dough without freezing. Week 3: bread obtained from frozen dough and stored for 3 weeks at −18 °C. Week 9: bread obtained from frozen dough and stored for 9 weeks at −18 °C. Volume decrease with respect to corresponding non-frozen samples, by frozen storage (%) after 9 weeks

9 weeks. Giannou and Tzia (2007) observed a great loss of loaf volume during the first months of dough frozen storage, but, thereupon, its variations were seriously reduced. It should be pointed out, however, as it was determined in a separate sensory evaluation study, that these samples were considered optimum breads by consumers, even when specific volume values were around 1.5 mL/g. Other authors also observed a negative effect of frozen dough on bread quality (Inoue and Bushuk 1992; Le Bail et al. 1999; Ribotta et al. 2001; Sharadanant and Khan 2003; Phimolsiripol et al. 2008).

The glucose oxidase enzyme caused the oxidation of wheat protein, thus strengthening gluten network. Results showed that bread obtained from frozen dough with higher levels of Gox presented larger volume than control bread from frozen dough. When Gox level was ≥0.005%, the percentage of bread volume reduction resulting from frozen dough was lower than the control (without enzyme; Table 1). These results indicated that the increase of dough strength by Gox addition partially counteracted the damage caused by dough frozen storage. Gox produced, mainly at the highest level, a decrease in bread volume values with respect to unfrozen control sample. Nevertheless, this enzyme enhanced the formation of larger volume pieces when compared with the control bread from frozen dough, especially after 9 weeks of storage. Gox inhibited the structural damage produced by ice crystal, since this enzyme improved gluten development and increased water absorption.

The addition of Pn to unfrozen dough produced breads with the largest volume. As these doughs were stored at −18 °C for 9 weeks, the specific volume also increased significantly with respect to control breads from frozen dough (Table 1). However, the percentage of bread volume reduction resulting from frozen dough storage was higher than that reached by the control bread made of frozen dough. This effect could be due to the decrease of pentosans size caused by Pn and concomitant release of large amounts of water. This water increased the number and size of ice crystals, which mechanically damaged gluten network. Berglund et al. (1991) and Ribotta et al. (2004), using scanning electron microscopy, confirmed the damaging effect of ice crystal formation in non-proofed and stored (−18 °C) dough. In spite of ice crystal negative effect, the volume of breads obtained from frozen dough with Pn enzyme for 3 weeks was the same as the volume of breads from control unfrozen dough without additives.

Transglutaminase enzyme promotes the formation of high weight molecular aggregates in gluten (Larré et al. 1998), modifying dough elasticity, and strength (Gerrard et al. 1998; Steffolani et al. 2008). The effect of TG on frozen dough was dependent on the level used. Only the addition of 0.1% of TG allowed us to obtain higher bread specific volume from frozen dough, as compared with control (Table 1). High levels of TG proved to have a similar effect to glucose oxidase, since they helped mitigate the damage produced by freezing, as volume decrease percentage was lower than control breads. The new isopetidic bonds introduced by TG could counteract damage on gluten network, improving CO_2 retention capacity during proofing, and thus obtaining a good bread quality. Huang et al. (2008) observed that high levels of TG increased the bread specific volume from frozen dough (stored for 5 weeks at −18 °C) and unfrozen dough. The differences between our results and those obtained by Huang et al. (2008) could be mainly due to differences in source and purity of transglutaminase enzyme (Yiming Fine Chemicals, Taixing, China in Huang et al.'s study and Apliena Ajinomoto, Japan in our study), and temperature of frozen storage (−30 °C). Low temperature diminished water mobility, and in consequence, the damage caused by ice crystal growth could be also lower. None of the three enzymes, at any of their levels, prevented the reduction in bread specific volume. However, they improved the BSV after 9 weeks of frozen storage, compared with frozen control samples, indicating that they could not completely counteract the effect of freezing but only mitigate it.

In general, breads obtained from frozen dough presented lower L^* and b^* values, and higher a^* value than breads obtained from unfrozen dough, indicating a decrease in crust lightness (darkness crust breads) with storage at −18 °C (Fig. 1). Similar results were obtained by Sharadanant and Khan (2003). Pentosanase enzyme decreased significantly L^* value of bread crust from frozen and unfrozen doughs, as compared with controls (without enzyme). This result could be due to the production of reducing sugars by enzyme action, which are substrates in the Maillard reaction.

Only the samples with higher TG and Gox levels (G3 and T3) counteracted the effect of frozen storage on crust color, since G3 and T3 breads from frozen dough presented a significantly higher L^* value and lower a^* value than controls (without enzyme), which is considered more desirable by consumers (Sharadanant and Khan 2003). This result could be due to the strengthening of the gluten network produced by higher TG and Gox levels, and the increase of water retention during baking, which is an unfavorable factor for Maillard reaction.

In baked products, crumb firmness and chewiness are commonly analyzed, as they strongly correlate with the consumer's perception of fresh bread (Faridi and Faubion 1990). The firmness of control bread crumb increased with dough frozen storage time (Table 2). These results are in agreement with those obtained by other researchers (Giannou and Tzia 2007; Phimolsiripol et al. 2008; Yi and Kerr 2009) who postulated that the increase in crumb

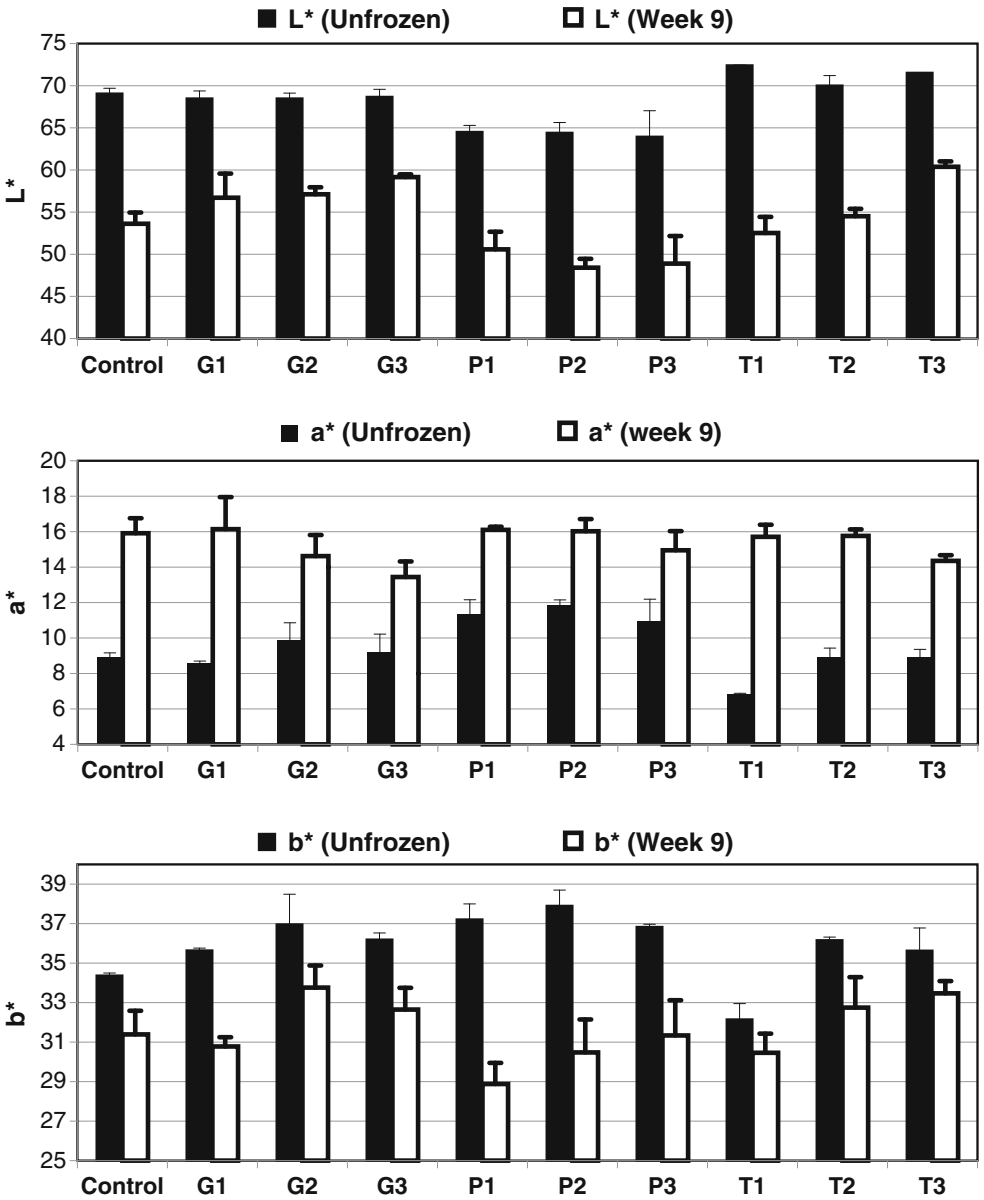


Fig. 1 Effects of enzymes on bread crust color (L^*, a^*, b^*) from unfrozen and frozen doughs G1, 0.001%; G2, 0.005%; and G3, 0.01% of glucose oxidase. T1, 0.01%; T2, 0.1%; and T3, 0.5% of transglutaminase. P1, 0.006%; P2, 0.012%; and P3, 0.018% of pentosanase. Unfrozen: bread obtained from dough without freezing. Week 9: bread obtained from frozen dough stored for 9 weeks at −18 °C

hardness could result from moisture loss during frozen storage, based on findings reported by He and Hoseney (1990) about the significant influence of moisture content on bread crumb firmness.

In general, crumb firmness of bread with enzyme addition also increased with increasing frozen storage time. These results had an inverse relationship with bread specific volume, so the increase in bread crumb firmness was probably related to a decrease in volume (Inoue and Bushuk 1992; Berglund and Shelton 1993).

Kenny et al. (1999) studied the effect of chemical oxidants on the quality of bread obtained from frozen dough and observed that ascorbic acid mitigated the effect of dough stored at freezing temperature on bread quality. In the present work, as enzymes are considered safe, glucose oxidase was used instead of oxidants. The G2 and G3 bread from frozen dough had lower crumb firmness than controls (without enzyme). The gluten networks of these samples were less damaged, and consequently, they increased crumb moisture retention, decreasing its firmness. In addition, the specific volume of these samples was higher than controls (without enzyme).

A similar effect on crumb firmness was observed in samples with Pn, but the highest specific volume developed by these breads produced low crumb firmness. Jiang et al. (2008) studied the thermo-stable xylanase effect on frozen pre-baked bread and observed that the enzyme increased specific volume and decreased crumb firmness, but the mechanism by which it mitigated the impact of freezing was not clear.

The addition of 0.1% of TG to frozen dough stored for 9 weeks produced breads with softer crumb than control.

Table 2 Effect of enzymes on firmness and chewiness of bread crumb

Sample	Firmness (g) unfrozen	Firmness (g) week 9	Chewiness (g) unfrozen	Chewiness (g) week 9
Control	652±17^{d}	794±33de	410±11^{d}	463±18^{cd}
G1	518±61^{c}	744±73^{cd}	335±40^{b}	435±27^{bc}
G2	631±49^{d}	706±27^{bc}	406±35^{d}	459±42^{cd}
G3	648±48^{d}	634±69^{ab}	427±12^{d}	387±46^{ab}
P1	405±40^{b}	766±70^{cd}	265±18^{a}	441±13^{c}
P2	289±13^{a}	650±42^{ab}	230±23^{a}	379±23^{a}
P3	321±38^{ab}	694±20^{bc}	235±25^{a}	391±18^{ab}
T1	522±58c	862±25ef	344±36bc	505±13d
T2	611±40cd	618±15a	402±6cd	393±12ab
T3	941±64e	879±52f	604±38e	552±25e

Different letters within a column indicate significantly different values ($p \leq 0.05$). G1, 0.001%; G2, 0.005%; and G3, 0.01% of glucose oxidase. T1, 0.01%; T2, 0.1%; and T3, 0.5% of transglutaminase. P1, 0.006%; P2, 0.012%; and P3, 0.018% of pentosanase. Unfrozen: bread obtained from dough without freezing. Week 9: bread obtained from frozen dough stored for 9 weeks at −18 °C

Kim et al. (2008) studied the addition of TG to frozen dough and observed that the enzyme caused a decrease of fresh bread quality, but it improved the bread quality obtained from frozen dough.

Crumb chewiness showed a similar trend to crumb firmness in breads from frozen dough (Table 2). In general, chewiness increased with dough storage time at −18 °C. Samples with Pn had the highest percentage of chewiness increase (60%) due to the freezing effect. However, P2 and P3 breads obtained from frozen dough had significantly lower chewiness than controls from frozen dough. Breads with 0.01% of Gox and 0.1% of TG were the ones with lower crumb chewiness after 9 weeks of dough storage at −18 °C.

At a macroscopic level, two different phases can be observed in bread crumb: an air phase and a solid phase representing cell wall. In a representative bread crumb sample, the solid phase is completely connected (Torquato 2000), although the air cell seems to be isolated. The volume of each phase fraction (solid and air; Mackenzie 1950; Ahmed and Jones 1990) and the nature of connectivity (Torquato 1998) determine the structure and, as a consequence, the mechanic properties of bread crumb. The type of gluten developed during the mixing process is very important for the conformation of crumb structure. Crumb structure density is affected by the insufficiency of native gluten polymer aggregation (Zghal et al. 1999). Thereby, freezing and enzyme effects on dough structure affect the crumb structure of resulting bread directly (Figs. 2 and 3).

Comparing the crumb structure of all breads from unfrozen dough, the higher levels of TG (T2 and T3) and Gox (G2 and G3) caused an increase in crumb uniformity (Fig. 2), so the resulting crumb was homogeneous and compact and had small air cells surrounded by thick protein–starch matrix. Consequently, these samples presented smaller cell area fractions than control breads (Fig. 3). The Pn enzyme caused the opposite effect: the crumb structure of P1, P2, and P3 (unfrozen dough) were less uniform (Fig. 2), had large cells, and thus cell area fractions were higher (Fig. 3). Zghal et al. (2000) stated that excessive breakdown of cell walls and gas cell coalescence, associated with weaker dough, leads to coarse and non-uniform crumb grain. Dough hardening, because of Gox and TG action, as was observed on dough resistance and extensibility, could explain the positive effect Gox and TG had on crumb uniformity. While, low uniformity of bread crumb with Pn was related to the formation of weaker dough.

The effect of freezing on crumb structure varied among samples. No big differences were observed in crumb structure among unfrozen and frozen dough breads. The main change was observed in crumb uniformity, where bread from frozen dough presented higher uniformity, probably due to the lower specific volume of this sample. Ribotta et al. (2001) observed that the longer the storage time in the frozen state, the higher the cell area fraction in the bread crumb. However, in the present work, significant differences were not observed in the CF of control breads before and after dough freezing. These differences could be principally due to differences in flour quality and bread-making method.

Freezing caused a decrease in crumb uniformity in samples with high levels of Gox (G2 and G3) and TG (T2 and T3), as compared with samples from unfrozen dough. On the contrary, bread with Pn from frozen dough presented greater crumb uniformity with respect to samples from unfrozen dough.

Comparing all samples from frozen dough, G2, G3, and T2 presented significantly higher CF than controls, and these results correlated ($p \leq 0.05$) positively with bread

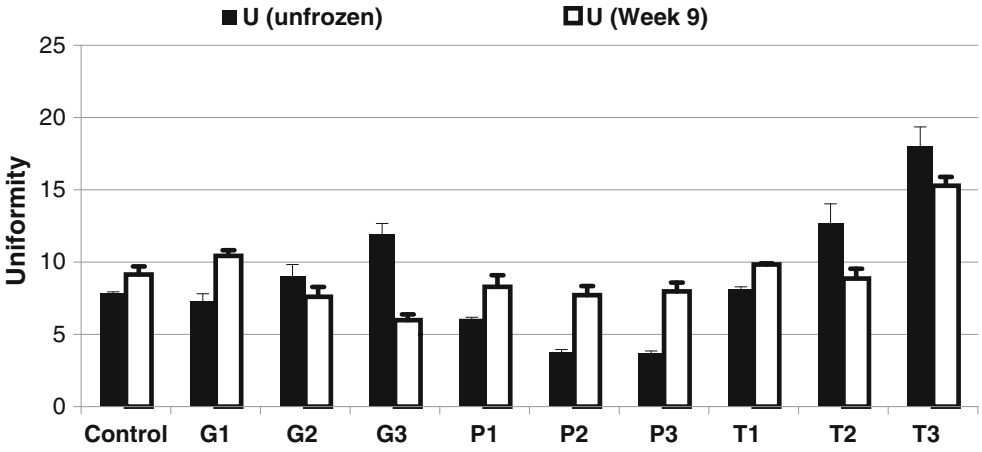


Fig. 2 Effect of enzymes on bread crumb uniformity from unfrozen and frozen dough. G1, 0.001%; G2, 0.005%; and G3, 0.01% of glucose oxidase. T1, 0.01%; T2, 0.1%; and T3, 0.5% of transglutaminase. P1, 0.006%; P2, 0.012%; and P3, 0.018% of pentosanase. Unfrozen: bread obtained from dough without freezing. Week 9: bread obtained from frozen dough stored for 9 weeks at −18 °C

specific volume (r=0.50) and negatively with crumb firmness (r=−0.66).

Bread images obtained from unfrozen and frozen doughs are shown in Fig. 4. The image of bread from unfrozen dough clearly shows that Pn enzyme produced a crumb with higher gas cells not uniformly distributed, whereas Gox and TG enzymes formed a closed crumb with a lower number of air cells uniformly distributed. Freezing modified the shape of bread pieces: after freezing, the dough becomes soft and weak producing wider and reduced height breads.

Effect of Enzymes on Frozen Dough Quality

All samples showed an increase in proofing time when bread dough had been frozen and stored at −18 °C for 9 weeks (Table 3). Similar results were presented in previous publications (Perron et al. 1999; Ribotta et al. 2001). These authors postulated that the increase in proofing time was due to the low CO_2 production by yeast and to the decreased ability of gluten for CO_2 retention. The enzymes used in our study did not fully mitigate this problem but the increase percentage of proofing time in samples with Gox was lower (21%) than values observed in control samples (36%), which would indicate that the enzyme acted on gluten network counteracting the damage caused by freezing to protein fiber.

Dough final volume developed during proofing could be a measure of its expansion capacity. The freezing effect on dough expansion capacity is presented in Table 3. Dough expansion capacity during proofing decreased with storage time at −18 °C in all samples. These results showed the same tendency obtained for bread specific volume. The Pearson correlation coefficient ($p \leq 0.05$) between dough expansion capacity and bread specific volume from unfrozen dough was r=0.92 and r=0.67 from frozen dough.

Dough adhesiveness and uniaxial extention assays were carried out by a Texture Analyser (Stable Micro Systems, Surrey, UK). Dough was prepared without yeast in order to evaluate the capacity of enzymes to counteract the mechanic damage caused by ice crystals on gluten structure. In addition, repetitive measurements of dough adhesiveness or extensibility could not be obtained because of yeast addition.

Adhesiveness was determined as the required force for raising the probe from dough surface. In all samples, including controls, the adhesion force increased with frozen

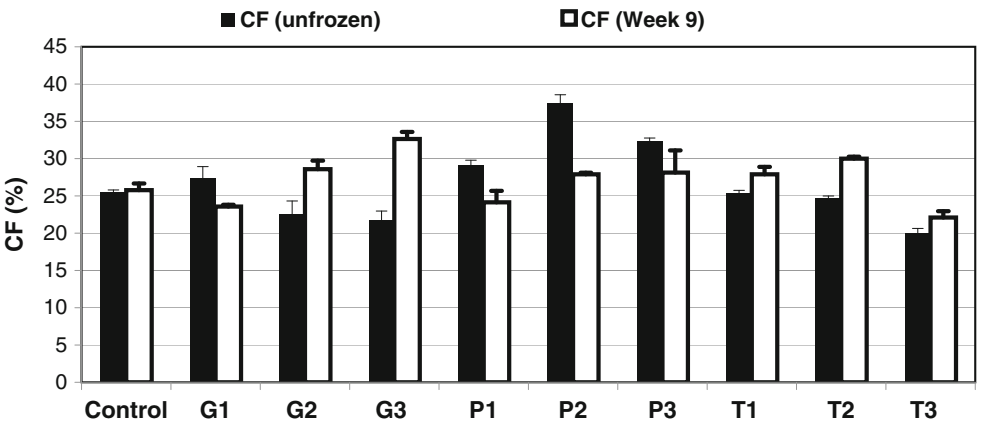


Fig. 3 Effect of enzymes on bread crumb cell area fraction (CF) from unfrozen and frozen dough. G1, 0.001%; G2, 0.005%; and G3, 0.01% of glucose oxidase. T1, 0.01%; T2, 0.1%; and T3, 0.5% of transglutaminase. P1, 0.006%; P2, 0.012%; and P3, 0.018% of pentosanase. Unfrozen: bread obtained from dough without freezing. Week 9: bread obtained from frozen dough stored for 9 weeks at −18 °C

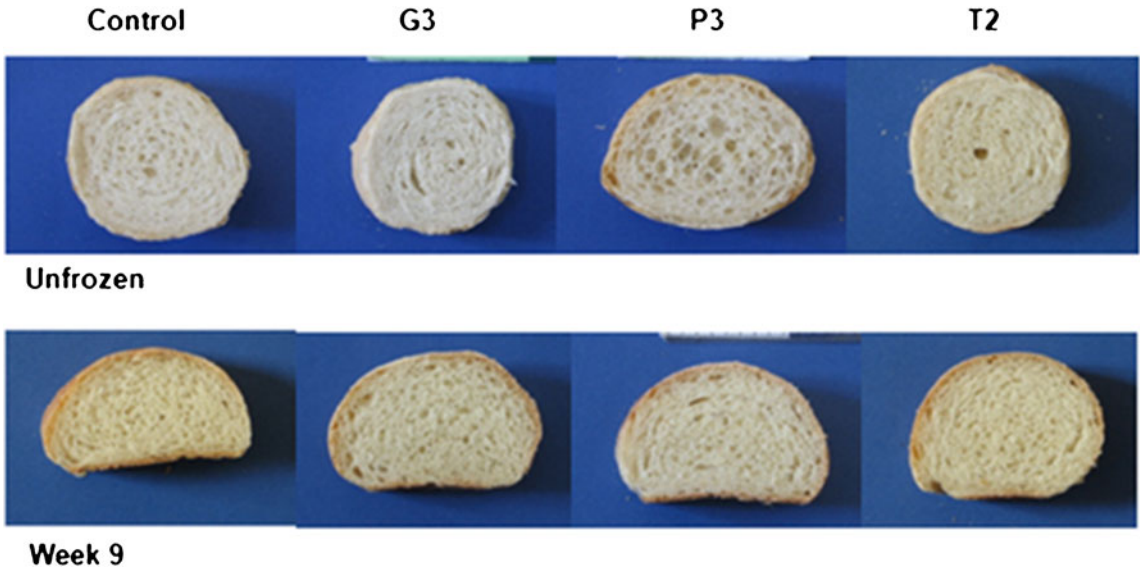


Fig. 4 Bread pictures from unfrozen and frozen dough. *Unfrozen*: bread obtained from dough without freezing. *Week 9*: bread obtained from frozen dough stored for 9 weeks at −18 °C. *Control*: bread without enzyme. *G3*: bread with 0.01% of glucose oxidase. *P3*: bread with 0.018% of pentosanase. *T2*: bread with 0.1% of transglutaminase

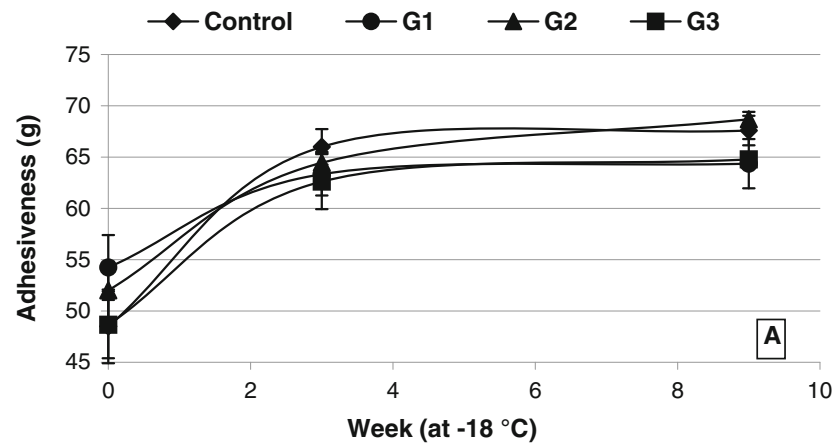
storage time (Fig. 5a–c), but the behavior was different according to the enzyme used. Adhesiveness increase could result from water redistribution during freezing, and after thawing, an increase in free water amount caused an increase in stickiness. These results were coincident with those presented by other authors (Angioloni et al. 2008), who determined dough adhesiveness by means of texture profile analysis and observed that these parameters went up with increasing dough frozen storage time. Yi and Kerr (2009) postulated that bread dough freezing and frozen storage could cause water separation from gluten network during ice crystal formation and that this water would remain more accessible at dough surface, thus increasing adhesiveness. In frozen and unfrozen samples, dough with Gox had similar adhesiveness than control dough (Fig. 5a). Also, all samples (control and with Gox) showed higher dough adhesiveness mainly during the first three weeks of storage at −18 °C, and after that, the increase was not significant.

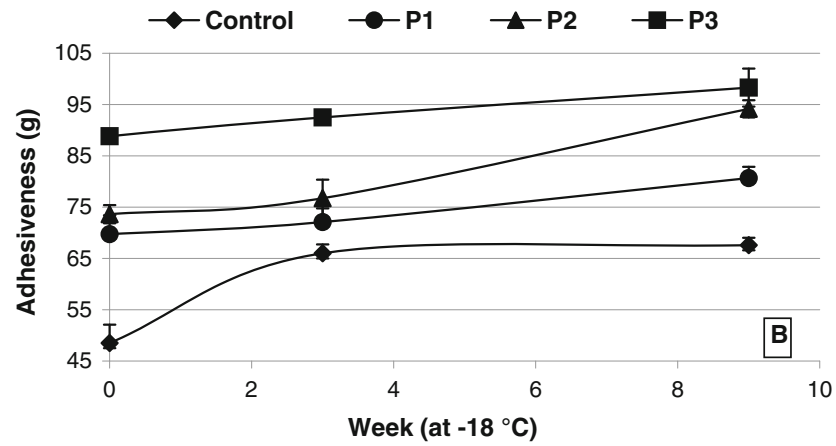
Dough adhesiveness increased with raising levels of Pn; these results were significant when compared with controls (without enzyme). This enzyme decreased pentosan size in dough, producing water redistribution as a result of which the dough surface was sticky to the touch. Comparing all samples from frozen stored dough, the samples with Pn presented higher adhesiveness than controls (Fig. 5b). Adhesiveness increase of control dough occurred mainly in the first 3 weeks of frozen storage; however, in dough with Pn, there was a mostly linear increase of adhesiveness with frozen storage time. The Pn enzyme left higher free water content to form ice crystals which, upon thawing, migrated to the dough surface increasing adhesiveness. Significant differences in dough adhesiveness were not observed in sample with TG or control, either in unfrozen or frozen doughs for 9 weeks. But, in contrast with control dough, in samples with TG, adhesiveness increase was linear with frozen storage time. Cauvain (2007) observed that dough at −18 °C is under the glass transition temperature; thereby frozen dough requires special storage to avoid dehydration. TG enzyme caused the formation of a new gluten network structure that modified water mobility at −18 °C, by which adhesiveness

Table 3 Proofing time and dough final volume developed during proofing

	Proofing time (min)		Final volume (mL)	
Sample	Unfrozen	Week 9	Unfrozen	Week 9
Control	105 ± 0^{bc}	143 ± 11^{bc}	169 ± 11^{bc}	136 ± 5^{ab}
G1	105 ± 0^{bc}	128 ± 11^{ab}	175 ± 7^{bc}	144 ± 5^{abc}
G2	105 ± 0^{bc}	128 ± 11^{ab}	167 ± 4^{bc}	151 ± 12^{c}
G3	98 ± 11^{b}	128 ± 11^{ab}	144 ± 2^{a}	156 ± 5^{c}
P1	105 ± 0^{bc}	128 ± 11^{ab}	195 ± 0^{de}	145 ± 0^{abc}
P2	98 ± 11^{b}	158 ± 11^{c}	205 ± 7^{e}	152 ± 11^{c}
P3	113 ± 11^{bc}	158 ± 11^{c}	200 ± 0^{e}	146 ± 5^{abc}
T1	110 ± 9^{bc}	143 ± 11^{bc}	182 ± 13^{cd}	135 ± 4^{ab}
T2	98 ± 11^{b}	120 ± 0^{a}	166 ± 5^{b}	147 ± 4^{bc}
T3	75 ± 0^{a}	120 ± 0^{a}	137 ± 7^{a}	132 ± 4^{a}

Different letters within a column indicate significantly different values ($p\leq0.05$). G1, 0.001%; G2, 0.005%; and G3, 0.01% of glucose oxidase. T1, 0.01%; T2, 0.1%; and T3, 0.5% of transglutaminase. P1, 0.006%; P2, 0.012%; and P3, 0.018% of pentosanase. Unfrozen: dough without freezing. Week 9: frozen dough stored for 9 weeks at −18 °C





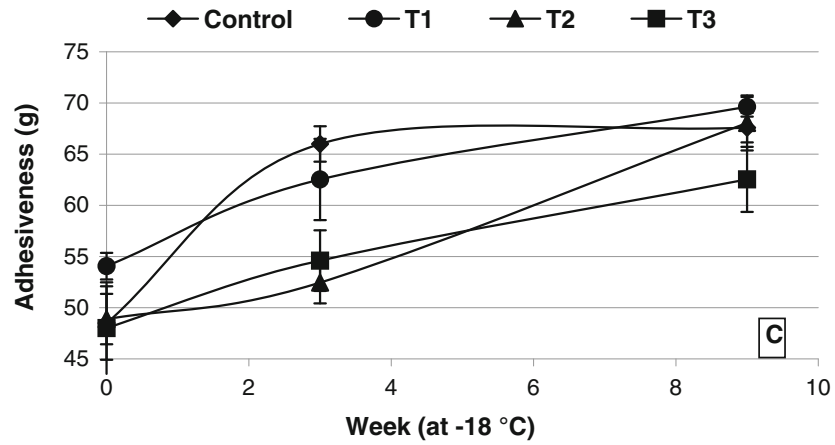


Fig. 5 Enzyme effect on dough adhesiveness variation with storage time at −18 °C. **a** Samples with glucose oxidase. **b** Samples with pentosanase. **c** Samples with transglutaminase. G1, 0.001%; G2, 0.005%; and G3, 0.01% of glucose oxidase. T1, 0.01%; T2, 0.1%; and T3, 0.5% of transglutaminase. P1, 0.006%; P2, 0.012%; and P3, 0.018% of pentosanase

increase with time was different from increase obtained in control dough (without the enzyme).

Dough extensibility determines the expansion ability of dough caused by CO_2 during proofing. An excessive extensibility results in soft and weak dough that collapse during proofing or baking (Sharadanant and Khan 2003). The resistance to extension is a measure of dough ability to retain CO_2. A lower resistance to extension results in a lower CO_2 retention capacity and lower bread specific volume; and a very high resistance to extension also results in lower bread specific volume because tough dough is not capable of developing the optimum height during proofing (Yi and Kerr 2009). In Table 4, maximum resistance to extension (Rm) and extension distance until breaking (Em) of doughs with and without enzyme, unfrozen and frozen, are shown. In the unfrozen samples, high levels of TG and Gox increased Rm and decreased Em significantly, as compared with control doughs (without enzyme). This effect is because the oxidant

Table 4 Enzyme effect on dough parameters of uniaxial extension from unfrozen and frozen dough

	Rm (g)		Em (mm)	
Sample	Unfrozen	Week 9	Unfrozen	Week 9
Control	50.0±0.3^{cd}	43.6±0.7^{c}	48.5±2.0^{c}	48.1±1.0^{c}
G1	43.7±4.2^{bc}	48.0±2.5^{d}	47.7±2.2^{c}	48.1±0.5^{c}
G2	57.8±1.1^{d}	47.2±0.6^{cd}	36.6±2.1^{b}	48.4±1.9^{c}
G3	69.9±3.6^{e}	54.9±0.1^{e}	28.7±2.4^{a}	41.8±2.1^{b}
P1	40.6±5.1^{bc}	37.6±2.3^{b}	53.0±7.4^{cd}	58.7±0.2^{d}
P2	34.4±3.1^{ab}	36.9±4.3^{b}	57.6±5.5^{de}	63.0±0.9^{e}
P3	28.2±2.6^{a}	32.5±1.4^{a}	63.3±3.0^{e}	70.3±2.4^{f}
T1	41.3±4.3^{bc}	45.0±0.3^{cd}	57.1±0.4^{de}	46.2±2.3^{c}
T2	79.3±7.5^{e}	58.1±2.0^{e}	31.3±2.6^{ab}	40.9±1.7^{b}
T3	72.7±0.1^{e}	71.4±1.7^{f}	28.9±0.3^{ab}	30.9±0.4^{a}

Different letters within a column indicate significantly different values ($p \leq 0.05$). G1, 0.001%; G2, 0.005%; and G3, 0.01% of glucose oxidase. T1, 0.01%; T2, 0.1%; and T3, 0.5% of transglutaminase. P1, 0.006%; P2, 0.012%; and P3, 0.018% of pentosanase. Unfrozen: dough without freezing. Week 9: frozen dough stored for 9 weeks at −18 °C. Rm (g): maximum resistance to extension. Em (mm): maximum extensibility

action of Gox enzyme led to a cross-link among proteins through disulfide and non-disulfide bonds. Strong and resistant dough, with a high P/L was obtained by high cross-linking between proteins. New isopeptidic bonds introduced by TG form aggregates are of a different nature from those formed by *S-S* bonds and, as a consequence, the dough has less extensibility and high resistance (Steffolani et al. 2010). On the contrary, Pn enzyme produced the opposite effect; it decreased Rm and increased Em, as compared with controls. The small size of pentosans produced by Pn promoted the increase of protein solubility in isopropanol due to changes of interactions among gliadins. These proteins are responsible for the viscous behavior of dough, leading to the formation of more extensible dough with a lower P/L (Steffolani et al. 2010).

Freezing had different effects on dough properties and depended on both, the type and the level of enzyme used. In control dough, frozen storage caused a decrease in maximum resistance to extension, indicating a weakening of gluten network. As the test was carried out without yeast, ice crystal formation caused a rupture in gluten matrix due to a mechanic effect, resulting in a discontinuous network, where starch granules were separated from protein network (Varriano-Marston et al. 1980; Havet et al. 2000; Giannou et al. 2003). Sharadanant and Khan (2003) and Yi and Kerr (2009) reported similar results; they observed that dough maximum extensibility increased with frozen storage time, whereas the maximum resistance to extension decreased. In samples with high levels of Gox (≥0.005%), Rm decreased and Em increased with longer times of frozen dough storage. Despite the damage, this enzyme helped to mitigate the freezing effect because G2 and G3 frozen doughs showed greater Rm values than control frozen dough (without enzyme). Dough extensibility increased with frozen storage time when Pn was added to the formulation, while resistance was not modified. Samples with TG did not show any change after frozen storage for 9 weeks, except for the intermediate level in which dough resistance to deformation was reduced and extensibility was increased. Comparing all doughs after storage for 9 weeks at −18 °C, G2, G3, T2, and T3 samples had higher resistance than control frozen dough, while P1, P2, and P3 doughs presented the lowest Rm. In contrast, dough extensibility was greater in samples with Pn than controls, and G3, T2, and T3 doughs had lower extensibility than controls.

Dynamic rheological properties were studied in dough prepared with yeast addition and stored for 9 weeks. The use of yeast in the formulation allowed us to analyze whether enzymes counteracted dead yeast negative effect during frozen storage.

All doughs analyzed presented a viscoelastic behavior with predominance in the elastic component, as G' was greater than G" in the whole frequency range (data not shown). In unfrozen samples, Gox and TG enzymes significantly increased the elastic module (G') value (Fig. 6). This effect was due to the high degree of protein

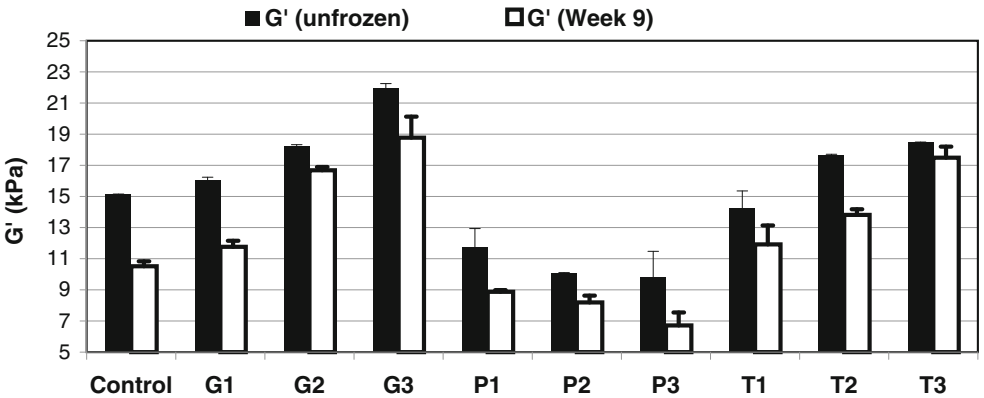


Fig. 6 Enzyme effect on dough storage module (*G'*) from unfrozen and frozen dough. G1, 0.001%; G2, 0.005%; and G3, 0.01% of glucose oxidase. T1, 0.01%; T2, 0.1%; and T3, 0.5% of transglutaminase. P1, 0.006%; P2, 0.012%; and P3, 0.018% of pentosanase. Unfrozen: dough without freezing. Week 9: frozen dough and stored for 9 weeks at −18 °C

cross-linking by enzyme action that caused the formation of strong and elastic doughs. On the contrary, Pn enzyme significantly decreased the elastic module, which led to less elastic and softer dough.

The frozen storage caused a decrease in G' in all samples (Fig. 6). The decrease percentage of control dough by frozen storage was~30%; this sample and P3 were the samples that had the greatest decrease percentage of G'. In the same way, Ribotta et al. (2003) reported a decrease of complex module (G^*) and dough elastic module (G') caused by freezing and frozen storage. Angioloni et al. (2008) obtained similar results, since they observed a decrease of G'and G", and an increase in tan δ of dough after 60 days of storage at −18 °C. Moreover, the mechanic effect of ice crystals on gluten network and dead yeast during dough freezing could cause a negative effect on dough viscolelastic properties. Kline and Sugihara (1968), Hsu et al. (1979), and Ribotta et al. (2003) confirmed that dough softening could be attributed to the release of some substances (e.g., glutathione) by dead yeast during freezing. Glutathione reduces the disulfide bond among gluten protein, and consequently, cross-linking of network decreases and so does its elasticity.

Samples with high Gox and TG contents presented the lowest decrease percentage of G' indicating that the enzyme action contributed to mitigate the negative effect of freezing on dough elasticity. Comparing all freezing and frozen stored samples, dough with high levels of Gox and TG presented greater G' values than controls (without enzyme; Fig. 6), indicating that doughs with enzymes were more elastic and showed a greater CO_2 retention capacity.

Conclusion

Although frozen doughs with Pn had higher damage percentage than control, bread resulting from frozen dough showed better characteristics than control from frozen dough. Further deterioration was mainly due to the decrease in pentosan size by enzyme action, causing water redistribution and leading to the formation of more and/or bigger ice crystals, which damaged the gluten network. However, breads with Pn from frozen dough had the best specific volume and the lowest crumb firmness; this could be due to the fact that smaller pentosans, as a result of Pn enzyme action, locate themselves in cell interfaces, increasing their expansion capacity without gas loss, and thus allowing a higher expansion during proofing.

Gox enzyme produced breads from frozen dough with better volume and better bread crumb textural characteristics than control. The oxidizing effect of the enzyme on gluten proteins helped to mitigate the damage caused by freezing and dough frozen storage. The higher protein cross-linking resulting from Gox effect counteracted the damage caused by ice crystals and the release of reducing substances by yeast cells killed by freezing. Therefore, the percentage of damage by freezing of dough with Gox was lower than the control, since the resulting dough was more elastic and resistant than control dough.

Only the intermediate level of TG produced breads from frozen dough with higher specific volume and lower crumb firmness than control. The new isopeptidic bonds introduced by TG in the gluten proteins helped to mitigate the damage caused by dough freezing. Although the structure of the gluten network formed by TG is different from the gluten network formed from disulfide bonds, the enzyme allowed us to obtain a dough with lower percentages of deterioration than control.

The enzyme that individually better counteracted the damage caused by dough freezing on bread quality was Gox; however, an optimization study would be valuable to define the best combination of these enzymes to improve bread quality.

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