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Effect of glucose oxidase, transglutaminase, and pentosanase on wheat proteins: Relationship with dough properties and bread-making quality

M. Eugenia Steffolani, Pablo D. Ribotta, Gabriela T. Pérez, Alberto E. León*

Facultad de Ciencias Agropecuarias, Universidad Nacional de Córdoba-CONICET CC509, 5000, Córdoba, Argentina

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ABSTRACT

Glucose oxidase (Gox), transglutaminase (TG), and pentosanase (Pn) were investigated for their effect on bread quality. The changes introduced in wheat protein by the action of these enzymes were analysed to explain dough behaviour. Gox treatment decreased free sulphydryl groups (*SHf*), increased glutenin macropolymer contents, and modified the electrophoretic pattern of protein fractions. Gox modified mainly albumin, globulin, and glutenin, forming large protein aggregates. These modifications explained the high strength of the dough and the low bread specific volume of samples with Gox. TG treatment modified solubility in SDS of protein and decreased glutenin macropolymer content. However, it formed large protein aggregates. The new cross-linking bonds introduced by this enzyme were different to S–S bonds and, consequently, the dough was less extensible and showed high resistance. Pn treatment increased water soluble pentosan content. Moreover, in these samples a tendency to increase *SHf* content was observed. In addition, Pn increased protein solubility in isopropanol, which indicates that the reduction of pentosans size decreases steric impediment of insoluble pentosans, thus increasing interaction among protein and making their extraction easier. These changes at the microscopic level allowed explaining the formation of softer dough and the production of higher specific volume in breads with Pn.

1. Introduction

The proteins of wheat flour are responsible for the formation of a viscoelastic dough capable of CO_2 retention during proofing and posterior baking (Lindsay and Skerritt, 1999; Shewry et al., 2001). The study of dough rheological properties is useful to predict attributes of the end product quality (Amemiya and Menjivar, 1992; Armero and Collar, 1998). In the last years, it has been postulated that the incorporation of covalent links among wheat protein improves the dough and the final product properties. Several preparations of commercial enzymes are used in the baking industry because these can improve different quality aspects of bread. In addition, the enzymes are a better alternative than chemical additives because they are regarded as safe.

The glucose oxidase (Gox) enzyme, 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). Vemulapalli et al. (1998) reported that H_2O_2, (produced by Gox) led to the formation of a soluble pentosan gel that increased dough consistency. During bread-making, the addition of Gox produced changes in dough texture and increased the bread volume (Martínez-Anaya and Jiménez, 1998). However, other authors (Risiah et al., 2005; Vemulapalli et al., 1998) did not observe great differences in crumb texture and did not obtain an increase in bread volume. Bonet et al. (2006) stated that the effect of Gox depends on the doses and the flour quality.

The microbial transglutaminase (TG) catalyzes the formation of intra or intermolecular ε-(γ-glutamyl) lysine isopeptide bonds. In a previous work, we found that transglutaminase introduced cross-linking mainly in high molecular weight glutenin subunits (HMW-GS), modifying the protein structure but increasing sodium dodecyl sulphate solubility (SDS) (Steffolani et al., 2008). Gerrard et al. (2000) observed a positive effect of TG on yeasted croissant and puff pastry. Other authors reported that the effect of TG is markedly dependent on the enzyme level and the quality of wheat flour utilised (Basman et al., 2002; Rosell et al., 2003).

Abbreviations: Gox, glucose oxidase; TG, transglutaminase; Pn, pentosanase; GMP, glutenin macropolymer; HMW-GS, high molecular weight glutenin subunits; LMW-GS, low molecular weight glutenin subunits; TP, total pentosan; WSP, water soluble pentosans; *SHf*, free sulfhydryl groups; SDS, sodium dodecyl sulphate; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; SEM, scanning electron microscopy.

* Corresponding author. Tel.: +54 351 4334105; fax: +54 351 4334116.
E-mail address: aeleon@agro.unc.edu.ar (A.E. León).

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The pentosans associated with flour proteins affect the formation of the gluten network by steric impediment (van Oort et al., 1995). Pentosanase (Pn) or xylanase enzymes hydrolyze arabinoxylans and arabinogalactans present in the flour. Xylanase releases water soluble arabinoxylans and also reduces their molecular weight (Courtin and Delcour, 2001). The use of xylanase improves the elasticity and the end product quality (Jiménez and Martínez-Anaya, 2001; Martínez-Anaya and Jiménez, 1997), decreases crumb initial firmness, and increases shelf life of bread (Haros et al., 2002) as well. However, Gil et al. (1999) had previously reported that xylanase did not modify hardening rate or crumb initial firmness. A xylanase thermostable recombinant produced a positive effect on bread quality, but only when used up to an optimum dose (Jiang et al., 2005). The knowledge of the mechanism by which the xylanase exerts its effects is still limited, probably because the flour pentosans change from one variety to the other, while enzyme specificity also changes according to the different substrates (Courtin and Delcour, 2001; Vardakou et al., 2003). The effect of this enzyme on the interaction among gluten proteins is still in discussion. Considering the contradictions found in the effects of different enzymes on bread quality, the objective of this work was to find changes introduced in wheat proteins by the action of enzymes, and hence explain dough behaviour and bread quality to make a contribution to the knowledge of enzyme mechanism in foodstuffs.

2. Experimental

2.1. Materials

Commercial wheat flour utilised for 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 × 5.7). Enzymes included glucose oxidase (Gox, Gluzyme mono BG from Novozyme, Denmark) 100,000 U/g activity (one 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 (one unit of TG is defined as the amount of enzyme that releases 1 μmol of hydroxamic acid in 1 min at 37 °C), endo-β-1,4-xylanase (Pn, Pentopan mono BG from Novozyme, Denmark) 2500 FXU/g activity (fungal xylanase units/g). The enzyme doses utilised were: Gox = 0.001% and 0.01%; TG = 0.01% and 0.5%; Pn = 0.006% and 0.012%. The doses of Gox and Pn used were recommended by the manufacturers and TG doses were selected according to previous publications. Wheat flour without enzyme was used as a control. All the reagent chemicals used were of analytical grade.

2.2. Preparation of freeze-dried dough

Flour and enzyme were mixed for 2.5 min with 58.5% water using a mixer (HR 1495, Philips, Argentina). Dough was rested for 1 h at 25 °C and then freeze-dried. Freeze-dried dough was stored at −20 °C until the analysis.

2.3. Total (TP) and water soluble pentosans (WSP)

Percentage of total pentosans and water soluble pentosans were analysed following the orcinol-hydrochloric acid method as described previously (Hashimoto et al., 1987). Freeze-dried dough dough (10 mg and 100 mg, respectively) was used for TP and WSP quantification.

2.4. Free sulfhydryl groups (SHf) determination

SHf were determined according to Bak et al. (2000) with modification of Hanft and Koeler (2006). Freeze-dried dough (50 mg) was mixed with 50 μL colour reagent (8 M urea, 3 mM EDTA, 1% SDS, 0.2 M Tris–HCl, and 10 mM 5,5-dithio-bis (2-nitrobenzoic acid), pH 8) and 1.5 mL buffer (8 M urea, 3 mM EDTA, 1% SDS, 0.2 M Tris–HCl, pH 8) for 25 min. The suspensions were centrifuged for 10 min at 3000 × *g* and the absorbance of supernatants was measured at 412 nm. The extinction coefficient used to transform absorbance values into concentration values was 13,600 M^{-1} cm^{-1}. Determinations were made in triplicate.

2.5. Glutenin macropolymer (GMP) isolation

GMP was extracted according to Skerrit et al. (1999). Freeze-dried dough (100 mg) was suspended in 1.5 mL of 1.5% (w/v) SDS and stirred for 1 h at room temperature. After centrifugation for 30 min at 15,600 × *g* at 20 °C, the supernatant was separated and stored at −20 °C for further analysis. Total protein content of precipitates was determined by the Kjeldahl method (N × 5.7) (AACC International, 2000) and expressed as % protein of GMP/g of freeze-dried dough.

2.6. Protein fractionation

Protein fractionation was made according to a modification of the sequential Osborne extraction method. Extraction was performed from 100 mg of freeze-dried dough. Each extraction step was carried out with constant stirring and centrifugation was done for 10 min at 1100 × *g* but 9400 × *g* in the last step. Sequential protein extraction was performed using 1 mL 5% w/v NaCl (F1), 70% v/v isopropanol (F2), 1.5% w/v SDS (F3) and sample buffer (0.063 M Tris–HCl, pH 6.8, 1.5% w/v SDS, 3% v/v β-mercaptoethanol, 10% v/v glycerol, and 0.01% w/v blue bromophenol; F4), for 2, 3, 8 and 4 h, respectively. Protein content of each fraction was determined by the Kjeldahl method (N × 5.7) (AACC International, 2000) and expressed as % of protein of each fraction/g of freeze-dried dough.

2.7. Protein fraction electrophoresis

The proteins from each fraction were analysed with electrophoresis assays. Electrophoresis was performed on 70 mm × 80 mm × 0.75 mm gels using a Mini Protein II cell (Bio-Rad Laboratories, Richmond, CA) according to Laemmli (1970). The fractions were resuspended in sample buffer (0.063 M Tris–HCl, pH 6.8, 1.5% w/v SDS, 3% β-mercaptoethanol, 10% v/v glycerol, and 0.01% w/v blue bromophenol). Separating gel of 12% and stacking gel of 4% acrylamide were used. Molecular weight standards were obtained from Bio-Rad (SDS-PAGE MW standards, Broad range, Bio-Rad). Electrophoresis was conducted at a constant voltage of 150 V until the front reached the end of the gel (≈90 min). Gels were stained with 0.25% Coomassie Brilliant Blue R in a methanol, water, and acetic acid (4:5:1 v/v) solution and were destained in the same solvent.

2.8. Dough rheological properties

The dough-mixing properties were examined with a farinograph (Brabender, Duisburg, Germany). Water absorption, dough development time and stability were determined according to AACC method 54-21(AACC International, 2000).

The rheological properties of the dough were determined with a TA.XT2i Texture Analyzer (Stable Micro Systems, Surrey, UK) using the SMS/KIEFFER rig for dough uniaxial extensibility measurements

and the Dobraszczyk/Roberts dough inflation system for dough biaxial extensibility measurements.

Doughs were prepared (flour basis) with standard formulation (1.8% salt, enzyme, and water optimum level). The ingredients were mixed for 2.5 min using a Philips HR 1495 mixer (Philips, Argentina) and rested for 15 min at room temperature. Dough (20 g) was pressed by the strip form and allowed to relax for 40 min. Ten strips by batch were placed on the texturemeter, trimmed and extended until their elasticity was exceeded and the dough broke. The dough strip was extended at 3.3 mm/s (Suchy et al., 2000). Resistance to extension (Rm, maximum resistance), extensibility (*E*, maximum extensibility), and area under the curve (*A*) were automatically calculated from the curves using the Texture Expert 1.22 software (Stable Micro Systems, Surrey, UK).

Biaxial extension was performed using the method described by Dobraszczyk (1997). Dough was inflated on a dough inflation system attachment mounted in the machine, at approximately constant strain rate of 0.1 s. Five dough disks were prepared with a special press (the disk was 27 mm thick and 55 mm diameter), were rested for 35 min, and then were subjected to an inflation assay.

2.9. Scanning electron microscopy (SEM)

The dough was prepared as described previously. Small portions of sample were cut with a razor blade and fixed in glutaraldehyde (1:30) for 2 h and embedded in a graded acetone series (25, 50, 75, and 80%) for 20 min at each gradation, then embedded in 100% acetone at three consecutive 20 min intervals to ensure full dehydration. Samples were then critical point dried. Critical point drying allows acetone removal in CO_2 without surface tension force that may distort the sample. Dehydrated samples were coated with gold particles for 4 min. The images were taken using a Jeol 35 CF (Japan) scanning electron microscope with a 6 kV acceleration voltage. The micrographs were taken using 1000 magnification.

2.10. Bread-making procedure and bread analysis

The dough formulation used included: 100% flour, 3% compressed yeast, 2.2% salt, 58.5% water (optimum level), and enzymes. 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 and the bulk dough was sheeted in a Mi-Pan vf roller (Mi-Pan, Córdoba, Argentina) having two rolls 50 × 12.7 cm^2. The dough was divided into 100 g pieces and moulded into a loaf shape (Braesi MB 350, Brazil). Then, pieces were proofed at 30 °C (96% relative moisture) up to its maximum volume increment and baked at 200 °C for 18 min. 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 ratio of finished bread. Texture profile analysis (TPA) was performed by using a TA.XT2i Texture Analyser (Stable Micro Systems, Surrey, UK) equipped with a 25-kg load cell. A cylinder probe of 2.5 cm in 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 cycle of compression under the following conditions: 1 mm/s crosshead speed and 40% maximum deformation. The texture profile parameters were determined using the Texture Expert 1.22 software (Stable Micro Systems, Surrey, UK). Bread crumb firmness and chewiness was calculated from a force–distance graph.

2.11. Statistical analysis

INFOSTAT statistical software (Facultad de Ciencias Agropecuarias, UNC, Argentina) was used to perform the statistical analysis. Determinations were done in duplicate. 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 $p < 0.05$).

3. Results and discussion

3.1. Effects of enzymes on pentosans

The effect of enzymes on total and water soluble pentosan contents is presented in Table 1. As was expected, the addition of enzymes did not modify total pentosan content of dough. Vemulapalli and Hoseney (1998) reported that the viscosity of the water soluble fraction extracted from fermented dough decreased with increasing levels of glucose oxidase, suggesting that the water soluble pentosans became less soluble or were degraded by the H_2O_2. However, the Gox enzyme did not modify water soluble pentosan content in comparison with the control sample.

No significant differences were observed in the content of water soluble pentosans among TG samples and control. This result indicated that the modifications introduced in proteins by transglutaminase did not change pentosan solubility.

As was anticipated, the content of water soluble pentosans increased in the samples with Pn, indicating the hydrolysis of water insoluble pentosans for enzyme action, namely the enzyme decreased pentosan size and facilitated their water extraction.

3.2. Effects of enzymes on SHf

SHf content of different samples is shown in Table 1. All samples with Gox had smaller *SHf* content than controls. The oxidant effect of Gox promoted the formation of disulfide bonds among protein and non-disulfide bonds among cysteine residues of protein and ferulic acid of arabinoxylans abating *SHf* (Stauffer, 2007). Hanft and Koeler (2006) observed a decrease of *SHf* only when glucose was also added to Gox; however, in the present study a decrease of *SHf* in the absence of glucose was observed.

No significant differences in *SHf* content were observed between TG 0.5% and the control sample. However, the lowest level of TG showed a higher *SHf* content, as compared to the control dough. TG catalyzes the formation of isopeptidic bonds, which modifies the three-dimensional structure of gluten protein. Therefore, the possibility of forming disulfide bonds depends on this structure, and the S–S formation and TG doses present there, are not directly related.

In the samples with Pn, a tendency to increase *SHf* content was observed, although only the highest enzyme level (0.012%) caused a significant change. These results could be due to a high non-covalent

Table 1
Effect of enzyme on content of total and water soluble pentosan (TP and WSP), free sulfhydryl group (*SHf*) and glutenin macropolymer (GMP) of freeze-dried dough.

Sample	TP (%)	WSP (%)	*SHf* (μmol/g)	GMP (%)
Control	3.57ab	1.35ab	0.53c	1.43c
Gox0.001	3.65ab	1.44b	0.35b	1.85d
Gox0.01	3.77b	1.31a	0.28a	1.91d
TG0.01	3.59ab	1.33a	0.84e	1.75d
TG0.5	3.58ab	1.37ab	0.53c	0.44a
Pn0.006	3.51a	2.06c	0.57cd	1.25c
Pn0.012	3.74b	2.07c	0.61d	1.42c

Letters within a column indicate significantly different values ($p < 0.05$).
Control: freeze-dried dough without enzyme, TG0.01 and TG0.5: freeze-dried dough with 0.01% and 0.5% of transglutaminase, Gox0.001 and Gox0.01: freeze-dried dough with 0.001% and 0.01% of glucose oxidase, Pn0.006, and Pn0.012: freeze-dried dough with 0.006% and 0.012% of pentosanase.

interaction between protein and pentosan (more interaction is expected as pentosan molecular size is decreased) which make the formation of disulfide bonds among glutenin proteins difficult and produces the *SHf* increase.

3.3. Effect of enzymes on wheat protein

In agreement with results reported by Primo-Martín et al. (2003), Gox enzyme increased protein content of GMP (Table 1), indicating the formation of large aggregates of protein insoluble in SDS which could be formed by disulfide and non-disulfide cross-linking. The effect of enzyme on the protein content of each solubility fraction is summarised in Table 2. The fraction extracted with NaCl solution (F1 of samples with Gox) corresponded mainly to albumins and globulins. While the F1 protein content of samples did not show significant differences, the electrophoresis pattern of 0.01% Gox showed a dark band of >200 kDa (Fig. 1A). This corresponds to large aggregates formed by non-disulfide cross-linking covalent bonds, since SDS-PAGE was made under reducing conditions. These bonds could be cross-linking between tyrosine residues of proteins. This result is in agreement with Risiah et al. (2005) and Vemulapalli et al. (1998) who reported that Gox modified albumins and globulins. Gliadin (F2) proteins were not affected by Gox (Table 2 and Fig. 1B) because of its low *SHf* content and because these form intra-chain disulfide bonds only. Several authors (Allen, 1999; Bonet et al., 2006; Risiah et al., 2005) indicated that a higher level of Gox was required to modify gliadin protein. Protein SDS solubility (F3) decreased with Gox addition and consequently F4 protein content increased (Table 2). The higher protein content in the last fraction indicated the occurrence of large SDS-insoluble aggregates formed by cross-linking disulfide and non-disulfide covalent bonds among proteins. Upon reduction, SDS-PAGE of F4 showed higher intensity in the complete pattern while the dark band at the top of the separating gel (non-disulfide cross-link covalent bonds) presented slightly higher intensity as compared to control samples (Fig. 1D).

The GMP protein content from dough decreased drastically and significantly when TG was added to 0.5%, although a lower dose increased the GMP content (Table 1). TG induced cross-linking into gluten proteins, but these new bonds caused structural changes in proteins, modifying their SDS solubility (Steffolani et al., 2008). TG catalyzes the formation of isopeptide bonds as well as the deamidation of glutamine residues where water molecules are used as acyl acceptors (Motoki and Seguro, 1998). This reaction occurs mainly when glutamine residues are predominant over lysine residues such as in gluten proteins. Glutamine is transformed to glutamic acid by increasing negative charge density; the increase of repulsive interactions among protein chains prevents a stable aggregation (Weegels et al., 1990) and this promotes an increase of solubility in SDS. However, when the protein solubility in different solvents was studied (Table 2), different results from the expected ones were observed. Samples with high levels of TG showed a reduction of protein solubility in NaCl solution as compared to the control. The action of TG on F1 promoted the formation of large aggregates insoluble in NaCl solution. In addition, molecules of albumin and globulin which have higher content of lysine could be participating in cross-linking with glutenin, decreasing NaCl solubility. SDS-PAGE protein patterns of this fraction showed an additional dark band at the top of the separating gel (Fig. 1A). Also a significant reduction of band intensity was found in samples treated with the higher level of TG in agreement with the protein content. These results indicated that large molecules were formed when TG was included in the formulation. Gerrard et al. (2001) reported similar effects on albumins and globulins by TG addition; besides, they found an increase of gliadin extraction. However, our results showed a decrease, significant at high dosis, in protein solubility in isopropanol (F2 of samples with TG).

The fraction extracted with SDS solution corresponds mainly to glutenins (F3), which decreased with the addition of 0.5% TG as compared to the control. This result was contradictory to the GMP content of the same sample (Table 1). In each step of the sequential extraction, the centrifugal force increased interactions between the protein chains, promoting the precipitation of aggregates. This result was corroborated with an additional experiment, where increasing hydration and centrifugation steps before GMP isolation were performed. Both control and TG samples had higher contents of GMP after successive steps of hydration and centrifugation; nevertheless the samples with high contents of TG had higher contents of GMP than the control (data not shown). SDS-PAGE of F3 of samples with TG showed in general a decrease of intensity of all bands mainly in HMW-GS (Fig. 1C). Greater participation of HMW-GS than LMW-GS in the formation of high molecular mass aggregates is due to the lower lysine content of LMW-GS (Mujoo and Ng, 2003). The last fraction was completely solubilised by sample buffer (F4) and the protein amount was higher in the TG 0.5% sample (Table 2). In agreement with several authors (Autio et al., 2005; Larré et al., 2000), the SDS-PAGE protein patterns of F4 showed large aggregates on top of the separating and stacking gel which correspond to those TG cross-linked proteins that did not enter the gel because the size of aggregates was larger than the size of pores (Fig. 1D).

GMP protein content of Pn and control samples did not show differences. However, Primo-Martín et al. (2003) reported that Pn increased protein content of GMP (Table 1). The Pn enzyme modified protein solubility in different solvents (Table 2). No significant differences were observed in F1 protein content of samples with Pn as compared to the control (Table 2). This result indicated that the higher content of water soluble pentosans did not modify NaCl protein solubility. On the other hand, the samples with Pn presented higher protein content in F2 as compared to the control, indicating that the lower pentosan size allowed extraction of larger amounts of protein with isopropanol. The reduction of pentosan size decreased steric impediment of insoluble pentosans and increased gliadin protein and alcohol–protein interactions, making protein extraction easier. On the other hand, a decrease of protein content in F3 and an increase of protein content in F4 were observed, due to Pn activity as compared to the control. This result indicated that a higher soluble pentosan content promotes associations between soluble arabinoxylan and protein through ferulic acid, decreasing the solubility of glutenin. In addition, the removal of insoluble pentosans would favour the increase of interactions among protein, also decreasing its solubility. Consequently, protein patterns of F4 of samples with Pn showed a higher intensity in all protein bands (Fig. 1D).

Table 2
Effect of enzyme on protein content of different solubility fraction.

Sample	F1 (%)[a]	F2 (%)[a]	F3 (%)[a]	F4 (%)[a]
Control	1.83b	3.84bc	3.32d	0.89a
Gox0.001	1.92b	3.74b	2.97c	1.39b
Gox0.01	1.89b	4.16cd	2.51a	1.85cd
TG0.01	1.85b	3.78b	3.33d	0.78a
TG0.5	1.50a	3.40a	2.76b	2.00d
Pn0.006	1.91b	4.46d	2.58a	1.51bc
Pn0.012	1.91b	4.33d	2.56a	1.41bc

F1: soluble protein in 5% w/v NaCl, F2: soluble protein in 70% w/v isopropanol, F3: soluble protein in 1.5% w/v SDS, F4: soluble protein in sample buffer.
Letters within a column indicate significantly different values ($p < 0.05$).
[a] Percentage of protein considering flour as 100%.

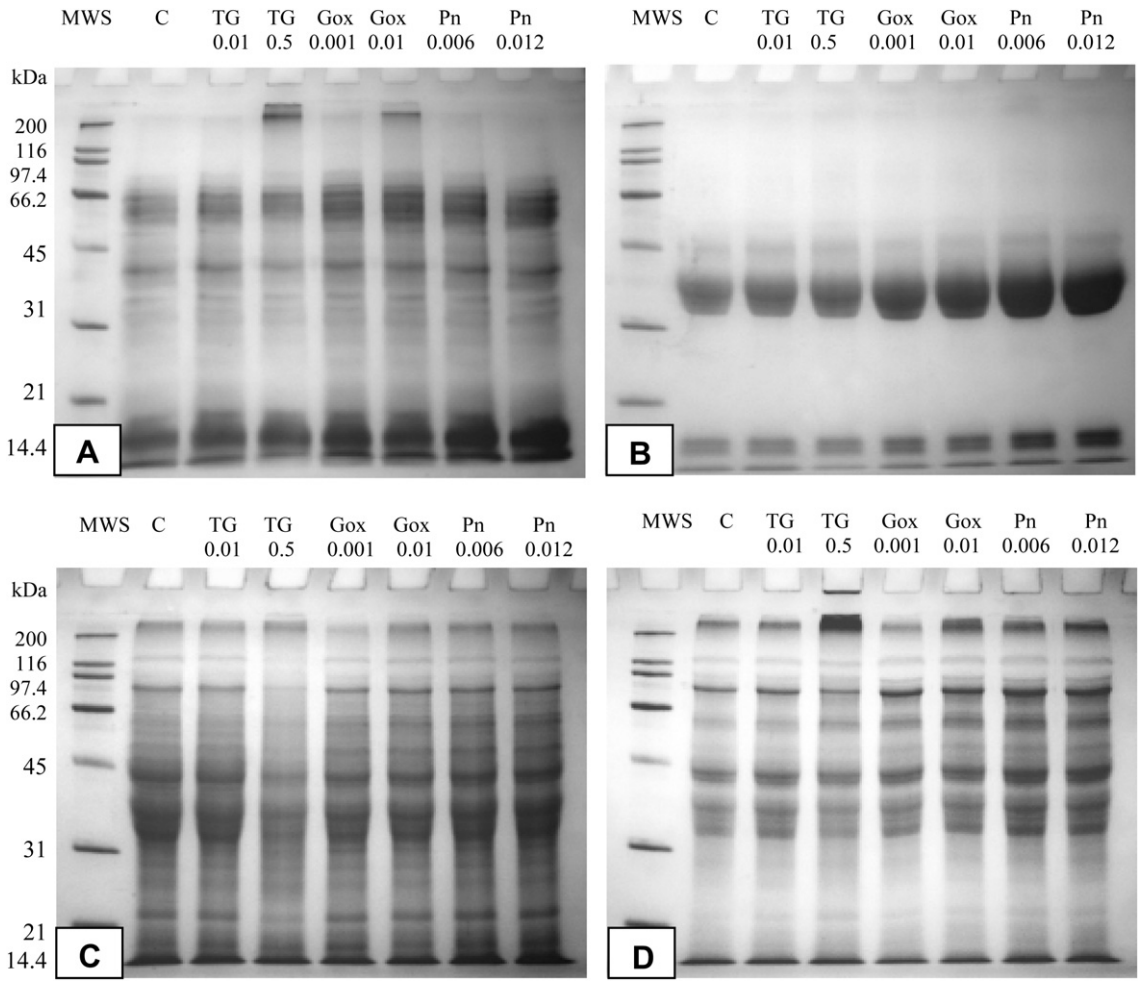

Fig. 1. Electrophoretic patterns of soluble protein in: (A) 5% w/v NaCl, (F1), (B) 70% v/v isopropanol (F2), (C) 1.5% w/v SDS (F3), (D) sample buffer (F4). MWS: molecular weight standard, Control: freeze-dried dough without enzyme, TG0.01 and TG0.5: freeze-dried dough with 0.01% and 0.5% of transglutaminase, Gox0.001 and Gox0.01: freeze-dried dough with 0.001% and 0.01% of glucose oxidase, Pn0.006 and Pn0.012: freeze-dried dough with 0.006% and 0.012% of pentosanase.

3.4. Effect of enzymes on dough rheological properties

The effect of enzymes on dough rheological properties is summarised in Table 3. No significant differences were observed in water absorption among Gox samples and their controls. Gox increased dough development time (DT) and stability (S) when enzyme dose increased. This result is in agreement with Bonet et al. (2006), who found that the addition of Gox did not significantly modify water absorption (WA) and promoted an increase in dough stability when it was over mixed. The highest Gox level increased maximum resistance to deformation (Rm) and decreased dough extensibility (E) as compared to control samples, leading to a kind of dough with similar characteristics to those of Argentinean wheat of group 1 (wheat for industrial bread-making with alveographic deformation energy of $340–600 \times 10^{-4}$ J) (Cuniberti, 2004). Similar trends were observed in biaxial tests, where the P value increased and the L value decreased when Gox was incorporated at 0.01%. This macroscopic effect results from the addition of high levels of Gox, which increased covalent cross-linking among proteins markedly and introduced S–S disulfide and dityrosine bonds into the gluten network. This result was confirmed by the SDS-PAGE profile and *SHf* content. However, the lowest level of Gox decreased

Table 3
Effect of enzymes on dough rheological properties.

Sample	WA (%)	DT (min)	S (min)	Rm (g)	E (mm)	A (g mm)	P (mm)	L (mm)	W ($J \times 10^4$)	P/L
Control	63.35a	1.88a	16.88ab	29.64ab	46.61cd	1086ab	188c	64ab	524a	3.0cd
Gox0.001	63.40a	3.88b	16.75a	35.18bc	47.92d	1337cd	161b	102c	709b	1.6ab
Gox0.01	63.35a	15.88c	20.00c	43.18d	36.48b	942a	269ef	68ab	839c	4.0efg
TG0.01	63.30a	2.00a	15.63a	30.73ab	49.03d	1186bc	239d	65ab	725b	3.7ef
TG0.5	63.60a	2.25[a]	19.00bc	55.30e	29.57a	1116ab	285f	66ab	865c	4.4fg
Pn0.006	63.30a	2.38[a]	15.63a	30.90ab	55.63e	1428d	170bc	78b	574a	2.3bc
Pn0.012	63.35a	2.00a	14.75a	27.82a	58.68e	1266bcd	123a	108c	544a	1.2a

WA: water absorption, DT: development time, S: stability, Rm: maximum resistance to deformation, E: maximum extensibility, A: area under the curve, P value: tenacity, L value: extensibility, W: deformation energy, P/L curve configuration ratio.
Letters within a column indicate significantly different values ($p < 0.05$).

P values and increased *L* values. Low Gox levels improved dough properties, since protein cross-linking was not as high as when 0.01% of Gox was used. Similar results were observed by several authors (Primo-Martín et al., 2003; Rosell et al., 2003).

Bauer et al. (2003) reported that high TG levels decreased dough development time and maximum consistency decreased. However, in this study, working with similar TG levels, no differences were found when compared to control samples. Uniaxial extension tests showed that Rm increased as the level of TG increased, indicating that TG strengthened the dough. In the same way, 0.5% TG dough had lower *E* than controls. These results agree with previous findings (Basman et al., 2002; Bauer et al., 2003). In biaxial extension, the *P* and *W* values increased as the level of TG increased, while the control dough had the lowest values. The properties of TG dough could be related to the increase of peptide cross-linking mainly in albumins, globulins and glutenins that led to a novel protein structure and changes in the interactions.

Farinograph parameters did not present differences between Pn and control samples, but Pn promoted a slight decrease of dough stability. Jiang et al. (2005) observed that thermostable recombinant xylanase decreased water absorption, development time, and stability, which indicated a decrease in dough strength. The samples with Pn presented higher *E* when the enzyme dose increased. These results were in agreement with parameters of biaxial tests, where Pn enzyme increased the *L* value and decreased the *P* value, indicating that Pn modified the aggregation properties of gluten protein. van Oort et al. (1995) suggested that the smaller size of pentosans led to a better aggregation of the gluten network, while Martínez-Anaya and Jiménez (1998) proposed that the smaller size of pentosans induced a redistribution of free water introducing modifications in the gluten network, which result in changes in dough viscoelastic properties. We stated that the higher extensibility of samples with Pn is related to the greater gliadin extraction in alcohol due to interaction changes among protein (mainly hydrophobic and hydrogen bonds). On the other hand, the changes in the interaction among dough components, mainly glutenin and water soluble pentosans, led to an increase of *SHf* content and, as a consequence, a softening of the dough.

3.5. Scanning electron microscopy (SEM)

Rojas et al. (2000) described dough as a continuous matrix (gluten network), in which starch granules are immersed. The microstructure of dough with high content of Gox and TG presented a more continuous and closed gluten network structure, as compared to the control dough (Fig. 2). The gluten fibrils were coarser indicating the formation of a stronger and more resistant network. Bonet et al. (2006) observed by Cryo-SEM that the Gox enzyme produced coarser and less oriented gluten fibrils with a greater number of larger-size pores, and that higher levels of Gox induced the formation of a discontinuous gluten network. In contrast, the microphotography of Pn dough (Fig. 2) showed a more open protein matrix and a greater number of pores than the control dough, where starch grains were more visible.

3.6. Effect of enzymes on bread quality

The effect of enzymes on bread specific volume and crumb texture is presented in Table 4. Gox decreased bread specific volume as added level increased. This result was a consequence of the increased strength of the dough. Despite no differences being observed in bread specific volume among control and Gox 0.001%, the latter showed less crumb firmness and chewiness than the control. However, the higher level of Gox had the same firmness and chewiness as the control. These results suggested that using weaker wheat flour than those used in this study, and lower Gox

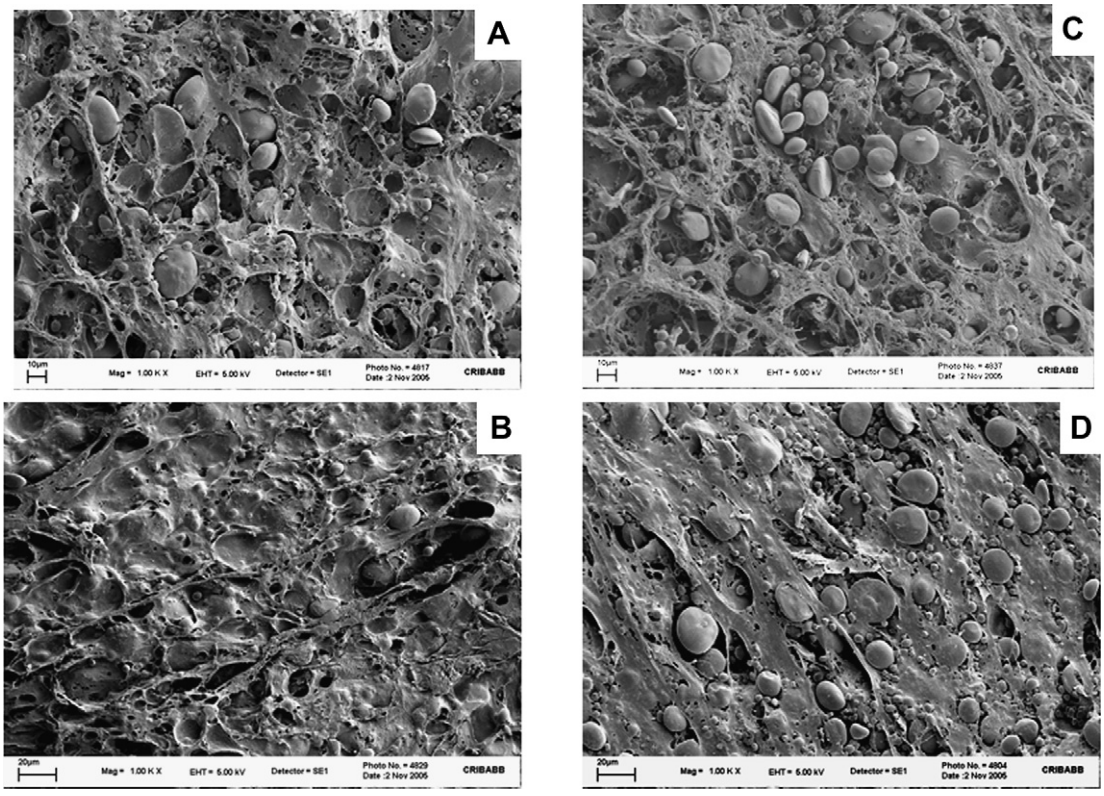

Fig. 2. Scanning electron microscopy of Control (A), Gox0.01 (B), Pn0.012 (C) and TG0.5 (D) doughs.

Table 4
Effect of enzyme on bread quality.

Sample	BSV (cm^3/g)	Firm. (g)	Chew. (g)
Control	4.67de	691ef	442e
Gox0.001	4.53d	626d	410d
Gox0.01	3.76b	652de	435de
TG0.01	4.71e	353a	249a
TG0.5	3.59a	1163g	738g
Pn0.006	4.98f	460b	309b
Pn0.012	5.24g	335a	223a

BSV: bread specific volume, Firm: crumb firmness, Chew: crumb chewiness. Letters within a column indicate significantly different values ($p < 0.05$).

levels could improve bread specific volume and texture. In a previous work, Bonet et al. (2006) suggested that Gox addition had different effects on bread volume and that these effects depended on the level used.

Bread specific volume decreased significantly with the addition of TG 0.5%. These results are coincident with the resistance to deformation, which was high enough to prevent the full development of the dough during fermentation. Basman et al. (2002) observed a negative effect of higher TG levels on loaf volume probably due to excessive cross-linking, causing an over-strengthening of the dough. Bread with 0.5% TG increased crumb firmness and chewiness; these results are related to low bread specific volume. However, the lower doses of TG reduced crumb firmness and chewiness as compared to the control. These results were coincident with Basman et al. (2002). As in the case of Gox, the TG effect is dependent on the dose used.

On the other hand, the addition of Pn allowed the development of higher bread specific volume. This result could be due to reinforcement of the cell structure by soluble pentosans (Jiménez and Martínez-Anaya, 2001; Martínez-Anaya and Jiménez, 1997). As a result of Pn activity, pentosans of lower molecular weight improved the expansion capacities of the cell without gas loss. Bread with Pn addition had a lower crumb firmness and chewiness. Similar results were published by Haros et al. (2002) who reported that the low initial hardness is a consequence of the high bread volume obtained. The *Pearson's correlation coefficients* among BSV and crumb firmness or chewiness were $r = -0.77$ and $r = -0.79$, respectively.

4. Conclusion

The effect of Gox modified wheat protein was mainly on albumins, globulins, and high molecular weight glutenin. The oxidant action of the enzyme led to a cross-link among proteins through disulfide and non-disulfide bonds. The formation of higher protein aggregates through disulfide bonds was corroborated by the decrease of *SHf* and the increase of GMP. The formation of non-disulfide bonds was proved by the presence of high molecular aggregates observed in electrophoresis under reducing conditions. Strong and resistant dough, with a high *P*/*L* was obtained by high cross-linking between proteins. Thus, the bread specific volume was low.

The new isopeptidic bonds produced by TG lead to the formation of greater aggregates that were retained in the stacking gel of SDS-PAGE. However, the cross-links caused structural changes in proteins, modifying their solubility in SDS. This could be due to the change in hydrophobic and electrostatic interactions and hydrogen bonding, which play an important role in dough structure. In addition, the new isopeptidic bonds introduced by TG form aggregates of a different nature from those formed by S–S bonds and, as a consequence, the dough has less extensibility. High levels of TG resulted in bread of low volume because the dough resistance was higher, and CO_2 pressure was not enough to achieve dough expansion.

The low 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 behaviour of dough, leading to the formation of more extensible dough with a lower *P*/*L*. In consequence, these samples developed breads with high volume and low firmness and chewiness.

The increase of protein cross-linking through isopeptidic bonds (by TG treatment) and S–S bonds (by Gox treatment) produced very strong dough and low bread volume when these enzymes were applied in Argentinean strong flour. Meanwhile the modification of interaction among gliadins and the increase of soluble pentosans promoted by Pn, increased dough viscosity and extensibility, leading to better retention of carbon dioxide and consequently improved bread quality.

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