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Kinetics of Transglutaminase-Induced Cross-Linking of Wheat Proteins in Dough

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The effects of TGase in dough after 15, 30, 45, and 60 min of resting time after mixing were studied with a Kieffer test. The resistance to stretching of control dough did not change greatly during the 60 min time period after mixing. In dough, TGase decreased extensibility and increased resistance to stretching and this change was already observed after the first 15 min (first measurement). The higher the enzyme dosage was, the higher the magnitude of the rheological change was. All of the doughs that contained TGase 3.8 or 5.7 nkat/g flour had a higher resistance to stretching and lower extensibility than control dough 15 min after mixing. Resistance to stretching clearly increased at a dosage of 5.7 nkat/g flour during the 15−60 min period after mixing. Extensibility increased in the control dough and in the doughs with a low enzyme dosage almost at the same rate. The evolution of air bubbles during proofing was determined with bright field microscopy and image analysis. In the presence of 5.7 nkat/g TGase, the fermented dough contained more of the smallest and less large air bubbles in comparison to the control dough. The effect of TGase and water content on the specific volume of the conventional and organic wheat bread was studied. Water did not have a significant effect on the specific volume of bread. TGase increased the specific volume of breads baked from organic flour only, when additional water (+10% of farinogram absorption) and a small enzyme dosage were used. Microstructural characterization showed that bread baked without TGase from conventional flour had a stronger protein network than that baked from organic flour. TGase improved the formation of protein network in breads baked from either normal or organic flour but at higher dosage caused uneven distribution.

KEYWORDS: Wheat; gluten; transglutaminase; rheology; microstructure; image analysis

INTRODUCTION

Wheat gluten is responsible for the baking performance, the unique rheological properties of doughs, and gas retention. Gluten is composed of extensible, viscous gliadins and rigid, elastic glutenins. Chemical oxidants are often used in baking, since they improve dough handling properties and increase fermentation stability and loaf volume. These chemical agents can be substituted by cross-linking enzymes, such as glucose oxidase (GOX), peroxides (POX), or transglutaminase (TGase). GOX catalyzes the oxidation of glucose to gluconolactone and hydrogen peroxide with subsequent formation of either disulfide bonds or dityrosine cross-links (*1*). The hydrogen peroxide generated by GOX may also lead to the oxidative gelation of arabinoxylans (*2*). It has been hypothesized that the oxidation product, hydrogen peroxide, is oxidizing gluten, since a similar effect has been observed with both galactose oxidase (*3*) and hexose oxidase (*4*). Hilhorst et al. (*5*) concluded that GOX not only increased the formation of covalent bonds in gluten but also affected the structure of cell wall components. It has been suggested that peroxidases promote the cross-linking of the protein network and arabinoxylans separately (*6*, *7*) and ferulic acid (FA) acts as a bridging agent. POX oxidizes FA, leading to the formation of feruloyl radicals, which can indirectly oxidize cysteinyl or the tyrosyl residues of proteins.

In contrast to oxidative enzymes, which also catalyze the network formation of arabinoxylans in doughs, TGase catalyzes the formation of inter- or intramolecular $\epsilon(\gamma$-glutamyl) lysine isopeptide bonds only in the protein fraction. By using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE) analysis, Larré et al. (*8*) showed that the TGase treatment increased the formation of large insoluble polymers. High molecular weight (HMW) glutenins were most affected, but some gliadins with a molecular mass of more than 70000 Da were also detected in the insoluble fraction after enzymatic treatment. Bauer et al. (*9*) also reported that the HMW subunits and α-gliadin are cross-linked by the TGase treatment. TGase

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Table 1. Chemical Analysis of the Flours

sample	protein content (% db, $N \times 5.7$)	ash content (% db)	gluten index
normal flour	13.7	0.72	29
organic flour	11.7	0.79	26

has been reported to cross-link water extractable albumins and globulins (*10*, *11*) and HMW glutenins (*10*−*12*).

Dynamic measurements as a function of the frequency and creep tests of TGase treated doughs showed a considerable reinforcement of the network (*8*). Creep recovery tests showed that the nonrecoverable strain decreased in a higher proportion than the recoverable strain in TGase-treated doughs (*13*) did. The network, however, remained transient. Larré et al. (*8*) suggested that the covalent intermolecular bonds modify the structure within the building blocks, which results in an increase in the number or the strengths of the nonpermanent cross-links between them.

Cross-linking enzymes have a great influence on the large deformation rheological properties of doughs. The mechanisms of the various enzymes differ greatly. The kinetics of cross-linking reaction in a dough are extremely important, although this has been studied less in relation to cross-linking enzymes. The objective of the present work was to study effects of TGase on dough extensibility and on resistance to extension after mixing as a function of time and to study the growth of gas bubbles during fermentation in relation to bread loaf volume and the microstructure of the protein network.

MATERIALS AND METHODS

Flours. Commercial bakers wheat flours were used. Conventional flour was from RaisioGroup plc (Raisio, Finland), and commercial organic flour was from Helsingin Mylly Ltd. (Järvenpää, Finland). Two replicates were made of the chemical analysis of the flours. The analyses of the flours used in the study are shown in **Table 1**.

Flour Analysis. The moisture content was determined according to standard method 44/15A, and the wet gluten content and gluten index were determined with method 38/12A (*14*). The protein content was determined by the Kjeldahl method (AACC standard 46-11A), and the ash content was determined by AACC standard 08-01. The optimal flour absorption was determined by recording farinograms (AACC 54-21).

Enzymes. TGase Activa WM (TG, Ajinomoto Inc., Japan) was purchased from Vesantti Oy (Finland). The TGase activity was determined according to Folk (*15*). The method is based on cross-linking of hydroxylamine with benzyloxycarbonyl-L-glutaminylglycine. The formed hydroxymate reacts further with $FeCl_3$ to form a ferric complex, which can be detected at 525 nm. The activity assay was carried out at 37 °C. The protein content was analyzed with a BioRad DC kit (Bio-Rad Laboratories, CA) using bovine serum albumin as the standard.

Fractionation of Wheat Gluten. First, 5 g of commercial gluten (80% protein, 7% fat; Sigma Chemicals, St. Louis, MO) was extracted twice with 100 mL of acetone, to remove the fat. The sample was then extracted twice with 50 mL of 0.5 M NaCl for several hours each time. The final extraction was carried out five times with 50 mL of 50% (v/v) ethanol. The residual precipitate was glutenin. The ethanol fractions were combined, and the sample was concentrated with a Rotavapor. Finally, the concentrated sample was freeze-dried. The residual precipitate was a gliadin fraction, which was not fractionated further.

Enzymatic Cross-Linking of Fractionated Gluten. The cross-linking experiments were carried out at a protein concentration of 3 mg/mL, at 40 °C in a 100 mM acetate buffer of pH 4.5. This relatively low pH was chosen to compromise protein solubility and enzyme activity. The reaction time was 17 h. The possible formation of cross-links was analyzed by SDS−PAGE according to Laemmli (*16*).

Table 2. Wheat Bread Formula

ingredients	quantity (g) conventional	quantity (g) organic
wheat flour	1000	1000
water	640, 655, or 670	590, 620, or 650
yeast	35	35
sugar	20	20
salt	15	15
shortening	40	40
TGase	0, 0.3, or 0.6	0, 0.3, or 0.6

Preparation of the Bread. The preparation of dough and bread from conventional and organic flours followed a standardized pan bread baking procedure as shown in **Table 2**. Thirty-five grams of yeast was stirred into different portions of the water and put into the kneader bowl. Sugar, shortening, and flour were added. The flour was mixed with the TGase. The doughs were kneaded with spiral kneader (Diosna SP 12 F, Dierks & Söhne, GmbH, Osnabrück, Germany) for 3 min at a low speed (100 rpm), followed by 4 min at high speed (200 rpm). Salt was added after 70 s of mixing at low speed. Six loaves of breads were baked for each experiment, and each set was made twice.

After the intermediate proof (15 min, 29 °C, 80% relative humidity), the dough was divided up into 6 × 400 g pieces. First, it was molded by hand and then by a cylindrical rounder (CR 59 K, M, G, Werner&Pfleiderer-Haton, The Netherlands) and a straight-line reverse-sheeting molder (BM 51-B, Werner&Pfleiderer-Haton). The molded dough pieces were placed in aluminum shells and proofed at 37 °C and 80% relative humidity for 55 min. The loaves were baked for 10 min at 240 °C, followed by 10 min at 220 °C (Rack Oven 9000, Sveba Dahlen AB, Sweden). Steam was added for 40 s during the initial baking phase. The breads were cooled to room temperature for 2 h before being weighed. The six loaves derived from each dough piece were termed as a batch. All measurements were taken using a batch as one collective unit. The loaf volume was determined by rapeseed displacement method (*17*).

EXPERIMENTAL PROCEDURES DESIGN

Statistical design, regression analysis of the results, and plotting of the response surface model (RSM) were performed using MODDE 4.0 (Umetri AB, Umeå, Sweden), comprehensive graphic Windows-based software for statistical experimental design, analysis, and interpretation. A two-level factorial design was chosen to simultaneously vary two ingredient parameters, water content and the dosage of TGase, relative to the chosen center point (water content 65.5% of conventional f.w. and 62% of organic f.w. and TGase content 0.3% of f.w.) to optimize water and TGase contents in wheat bread. The response to variations in water and TGase was measured as the specific volume of the bread. The RSMs were estimated by multiple linear regression [partial least squares (PLS)] for the 21 experiments in the central composite face-centered design. The center point made it possible to estimate the pure error of the analyses, which was used to predict whether the models gave significant lack of fit (*18*). The reliability of the models was evaluated by calculating the R^2 and Q^2 values for each model, where R^2 is the variation of the response explained by the model and Q^2 is the fraction of the variation of the response that can be predicted by the model. Q^2 should be >0.5 if conclusions are to be drawn from the model (*19*).

Uniaxial Extension Tests. Uniaxial extension studies were performed at 30 °C with a Kieffer extensibility rig fitted on the Texture Analyzer (Stable Microsystems Ltd., United Kingdom) equipped with a 5 kg loading cell. A roll of the dough was put on the lower lubricated plate and compressed with the top lubricated plate to prepare dough samples for the measurement.

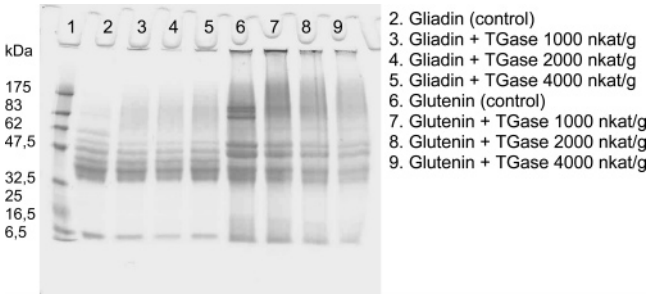

Figure 1. SDS–PAGE of gliadin and gluten crossliked by TGase.

After 10 min of resting (the dough had relaxed) at 30 °C, the dough sample was placed on the lower plate of the extensibility rig and the first measurement was recorded after 15 min of resting time after mixing. The deformation rate was 32 mm/min. Three different doughs were prepared, and four different measurements were performed for each dough.

Microscopy and Image Analysis. The control dough and a dough containing 5.7 nkat/g TGase were proofed at 30 °C and 80% humidity for 20 and 40 min. The yeast doughs were frozen and freeze-dried. The samples were embedded in plastic as reported by Räsänen et al. (*20*). The samples were examined with an Olympus BX-50 microscope (Tokyo, Japan). Micrographs were obtained with a SensiCam PCO CCD camera (PCO, Kelheim, Germany) and analyzed with the AnalySIS 3.0 image analysis program (Soft Imaging System, Münster, Germany). For doughs after mixing, the area analyzed was approximately 50 mm^2 per bread, and for doughs that had been fermented for 20 and 40 min, the area analyzed was approximately 200 mm^2 per bread. Two replicate doughs and two pieces of each dough were analyzed.

Pieces of bread crumbs (0.5 cm) were taken from the middle of the loaf, embedded in 1% agar, fixed in 1% glutaraldehyde in a 0.1 M phosphate buffer, pH 7.0, dehydrated with ethanol, and embedded in hydroxyethyl methylacrylate as recommended by the manufacturer (Historesin, Leica, Heidelberg, Germany). Sections were cut 4 μm thick in a Leica rotary microtome HM 355 (Heidelberg, Germany) using a steel knife. The sections were transferred onto glass slides and stained with acid fuchsin (*21*). The samples were examined with an Olympus BX-50 microscope (Tokyo, Japan). Micrographs were obtained with a SensiCam PCO CCD camera (PCO).

RESULTS AND DISCUSSION

Cross-Linking of Gliadin and Glutenin Fractions by TGase. The fractionated gliadins and glutenins were cross-liked by TGase using different enzyme dosages. The cross-link formation was followed by SDS−PAGE (12% acrylamide Tris-HCl gel) (**Figure 1**). The glutenin fraction could be cross-linked more easily than the gliadin fraction. The SDS−PAGE analysis indicated that the HMW protein subunits (85, 109, and 126 kDa) in glutenin disappeared totally due to TGase. In addition, HMW products unable to penetrate the gel were formed (**Figure 1**, lanes 7−8) (**Table 3**). Furthermore, the lower molecular weight (LMW) subunits were cross-linked, especially in samples with a high enzyme dosage. As discussed by Larré et al. (*8*), the differences in reactivity between HMW and LMW are presumably due to the higher lysine content in HMW. The gliadin fraction was not very reactive, as only the 59 kDa subunit disappeared in the samples treated with TGase. Gliadins are known to contain a lower amount of lysine as compared to glutenins.

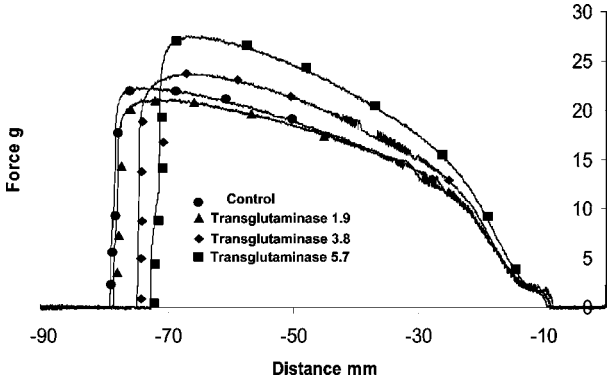

Figure 2. Typical force–deformation curves for doughs without and with TGASE. Mean values of seven replicates. The dosages were 1.9, 3.8, and 5.7 nkat/g flour.

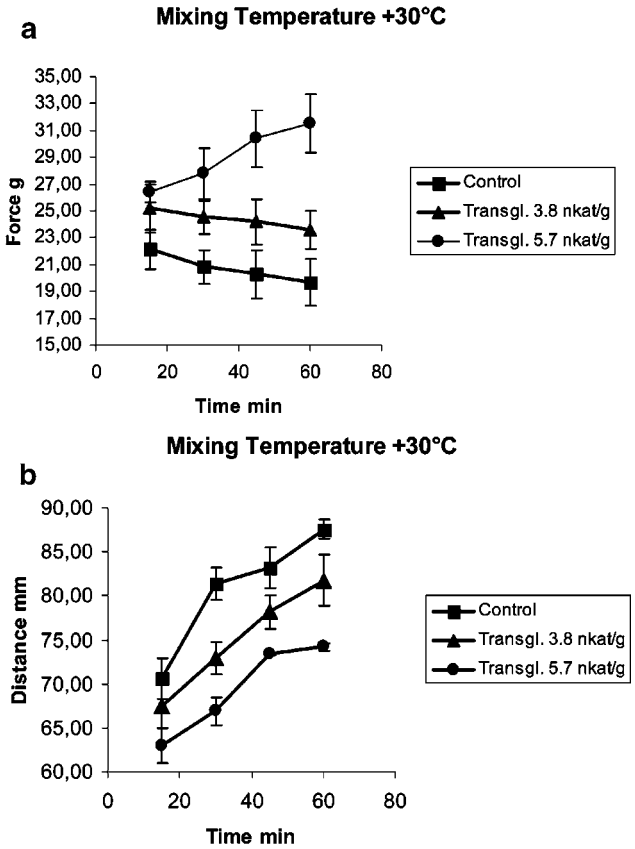

Figure 3. Effect of dough resting time after mixing on (**a**) resistant force and (**b**) the extensibility of dough. Mean values ± standard error of seven replicates.

Rheological Measurements. A typical force−distance curve for doughs is presented in **Figure 2**. The addition of TGase at 3.8 and 5.7 nkat/g increased resistance to stretching and decreased the extensibility in relation to control dough (**Figure 3a**,**b**) and a dough containing 1.9 nkat TGase/g flour (results not shown). The resistance to stretching was higher for doughs containing 3.8 and 5.7 nkat TGASE/g flour, when the doughs were kept at 30 °C for 15 min after mixing. The resistance to stretching of the dough containing 5.7 nkat TGase/g flour greatly increased during the 60 min resting period at 30 °C, whereas the force slightly decreased in the control dough and doughs with lower enzyme dosage (**Figure 3a**). The extensibility of all of the doughs increased during resting at 30 °C (**Figure 3b**).

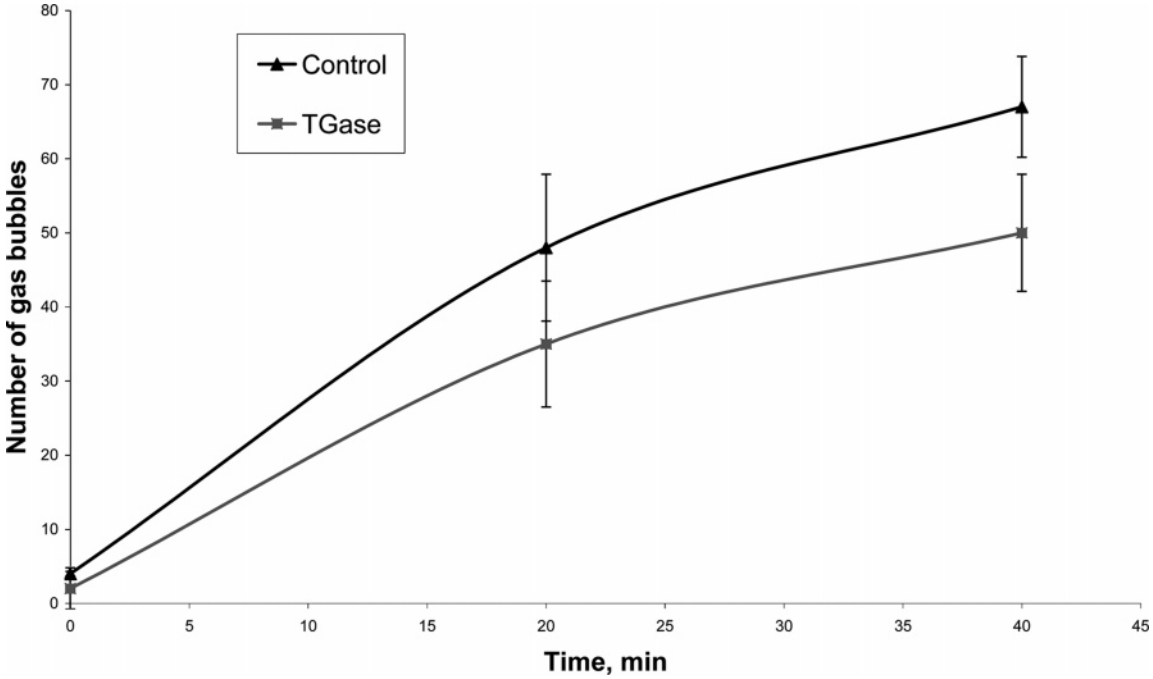

Figure 4. Number of largest air bubbles (0.01–0.05 mm^2) after mixing and 20 and 40 min of proofing time. Mean values ± standard error of four different sections.

The higher the dosage of TGase, the lower the extensibility was. The enzyme dosage that we used was clearly higher than that used by Bauer et al. (*13*), who did not find that it had any effect of Tgase on the dough system. They, however, did show that TGase decreased gluten extensibility and increased resistance to extension, which agrees with the results presented here. With these large deformations, the resistance presented by the covalent cross-links dominates. According to Larré (*8*), TGase improved the connectivity of the gluten network. As discussed by Larré et al. (*8*), TGase treatement keeps the dough viscous (extensible), despite additional covalent cross-links being created.

Growth of Air Bubbles during Fermentation. The number of air bubbles of different sizes were determined in doughs before and after the 20 and 40 min of proofing time by microscopy and image analysis. After 20 and 40 min of proofing time, the average area of pores was higher in the control dough (results not shown). The higher resistance to stretching and the lower extensibility of the dough containing TGase decrease the growth of the largest air bubbles (**Figure 4**). The control dough, which was fermented for 20 min, contained more of the largest and less of the smallest air bubbles in comparison to the TGase-treated dough (**Figure 5a**,**b**). During dough proofing and baking, the growth of gas bubbles determines the expansion of the dough and, thus, the volume of the bread. This is particularly the case with large bubbles, which will grow at a higher rate than smaller bubbles, since less pressure is needed for the expansion of large bubbles.

Baking Experiments. The range of water and TGase content was chosen according to the preliminary baking experiments using normal and extreme values. The design used in this investigation could accommodate the factors x_1, x_2, x_1^2, x_2^2, and x_1x_2. To determine the optimum of TGase and water content in these breads, each factor was used at three scaled levels as follows: (i) conventional and organic x_1: −1 (TGase 0% of f.w.), 0 (TGase 0.3%), +1 (TGase 0.6%); (ii) conventional x_2: −1 (water 64% of f.w.), 0 (water 65.5%), +1 (water 67%); and (iii) organic x_2: −1 (water 59% of f.w.), 0 (water 62%), +1 ((water 65%).

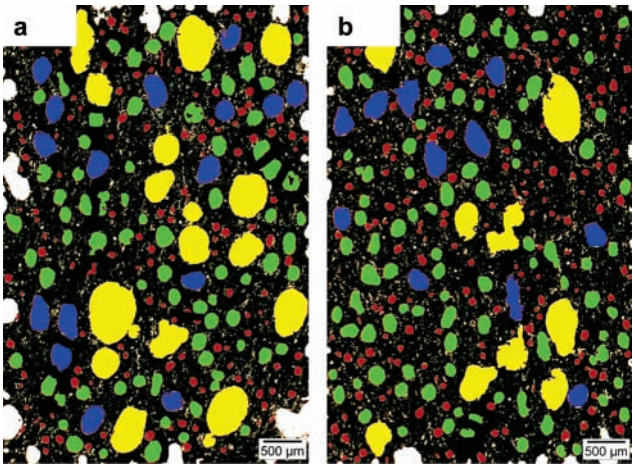

Figure 5. Representative micrograph of a fermented (**a**) control dough and (**b**) TGase (5.7 nkat/g) containing dough after 20 min of proofing. The largest bubbles (0.01–0.05 mm^2) are yellow, the next largest bubbles (0.005–0.01 mm^2) are blue, the second smallest bubbles (0.001–0.005 mm^2) are green, and the smallest bubbles (0.0001–0.001 mm^2) are red.

The coefficients in the analysis were calculated by the least squares method, and a 95% confidence interval on the values of the parameters was assigned. These coefficient values were used to predict the response surface. The standard error and lack of fit were low because of the reproducibility of the replicates (**Table 4**).

The specific volume of the breads with different water and TGase contents varied with a range of 3.3−4.0 cm^3/g; yet, the reproducibility of the replicates was very good. Complete RSMs, including the 21 experiments in the design, were estimated. The results from the measurements were evaluated statistically by PLS. The achieved mathematical model, expressed in unscaled variables, was

Table 3. Densitometer Analysis from the SDS–PAGE Showing Cross-Linking of Gliadin and Glutenin by TGase (**Figure 1**)

protein	gliadin				glutenin			
subunit	lane	lane	lane	lane	lane	lane	lane	lane
(kDa)	2	3	4	5	6	7	8	9
126					100			
109					100			
85					100			
59	100							
52	100	82	82	88				
51					100	80	57	49
47	100	88	75	88				
45					100	84	61	53
41	100	92	88	100				
38	100	89	83	94	100	92	67	53
35	100	89	86	100	100		62	50

$$y_{\text{calcd}}\ (\text{specific volume of the conventional bread}) = -0.57 + 0.5x_1 + 0.07x_2 - 1.67x_1x_1$$

$$y_{\text{calcd}}\ (\text{specific volume of the organic bread}) = 3.78 + 0.57x_1 - 0.002x_2 - 1.08x_1x_1 + 0{,}05x_1x_2$$

The TGase (x_1) significantly ($P < 0.05$) reduced the specific volume of the bread baked with conventional wheat flour, while water did not have a significant effect on specific volume. The model showed no significant lack of fit, and the estimation of the models gave $R^2 = 0.77$ and $Q^2 = 0.56$ for a specific volume of the breads. The water (x_2) and interaction term of water and TGase (x_1x_2) significantly ($P < 0.05$) increased the specific volume of the organic bread. This means that when water content and Tgase were increased simultaneously, the volume increased. Square terms of TGase (x_1x_1) significantly ($P < 0.05$) decreased the specific volume. The specific volume decreased when the TGase content was below or over the optimum range. The model showed no significant lack of fit, and the estimation of the models gave $R^2 = 0.73$ and $Q^2 = 0.54$ for a specific volume of the organic bread.

Two-dimensional presentations of the response surfaces are given in **Figure 6a** for a specific volume of the bread baked with conventional flour and **Figure 6b** for bread baked with organic flour. The maximum specific volume (optima) of the

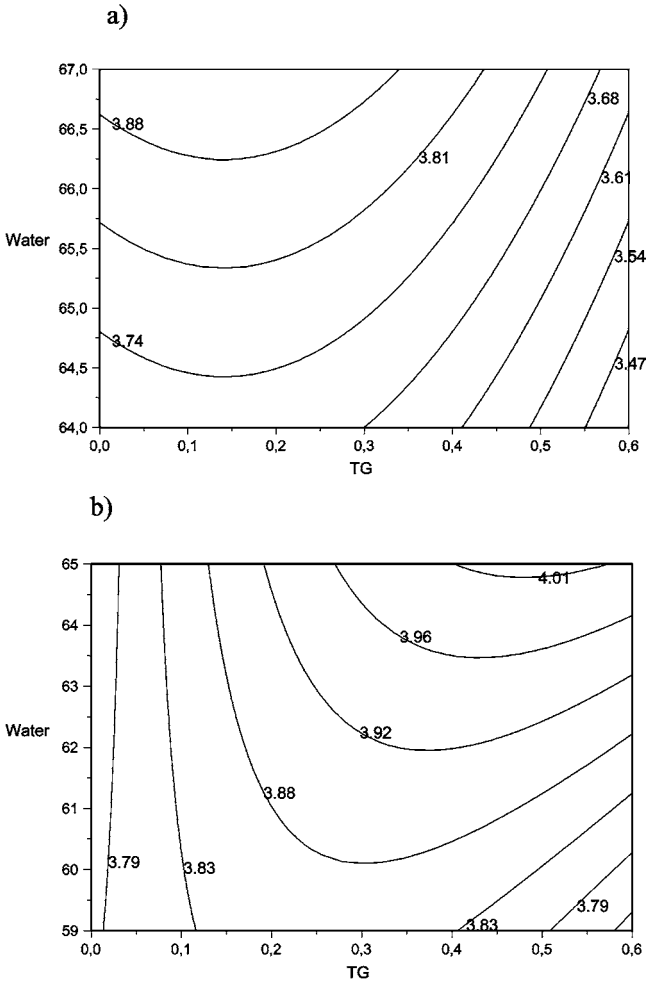

Figure 6. Two-dimensional response surface for the specific volumes of breads (**a**) breads baked with conventional flour and (**b**) breads baked with organic flour. All isobars are values of specific volume in cm^3/g bread. Water and TGase contents are % of f.w.

Table 4. ANOVA Tables of the Specific Volume of Conventional and Organic Breads

spec volume	DF	SS	MS (variance)	*F*	*p*	SD
			conventional			
total	10.00	140.140	14.014			
constant	1.00	139.876	139.876			
total corrected	9.00	0.264	0.029			0.171
regression	3.00	0.204	0.068	6.800	0.023	0.261
residual	6.00	0.060	0.010			0.100
lack of fit (model error)	4.00	0.053	0.013	4.000	0.210	0.115
pure error (replicate error)	2.00	0.007	3.333e-03			0.058
$N = 10$	$Q2 = 0.5587$		CondNo = 2.522			
DF = 6	$R2 = 0.7727$		Y-miss = 0			
comp = 2	$R2$Adj = 0.6591		RSD = 0.1000			
			ConfLev = 0.95			
			organic			
total	21.00	313.960	14.950			
constant	1.00	313.741	313.741			
total corrected	20.00	0.218	0.011			0.104
regression	4.00	0.160	0.040	11.025	1.734e-04	0.200
residual	16.00	0.058	3.633e-03			0.060
lack of fit (model error)	4.00	0.020	4.998e-03	1.573	0.244	0.071
pure error (replicate error)	12.00	0.038	3.178e-03			0.056
$N = 21$	$Q2 = 0.5354$		CondNo = 2.630			
DF = 16	$R2 = 0.7338$		Y-miss = 0			
comp = 2	$R2$Adj = 0.6672		RSD = 0.0603			
			ConfLev = 0.95			

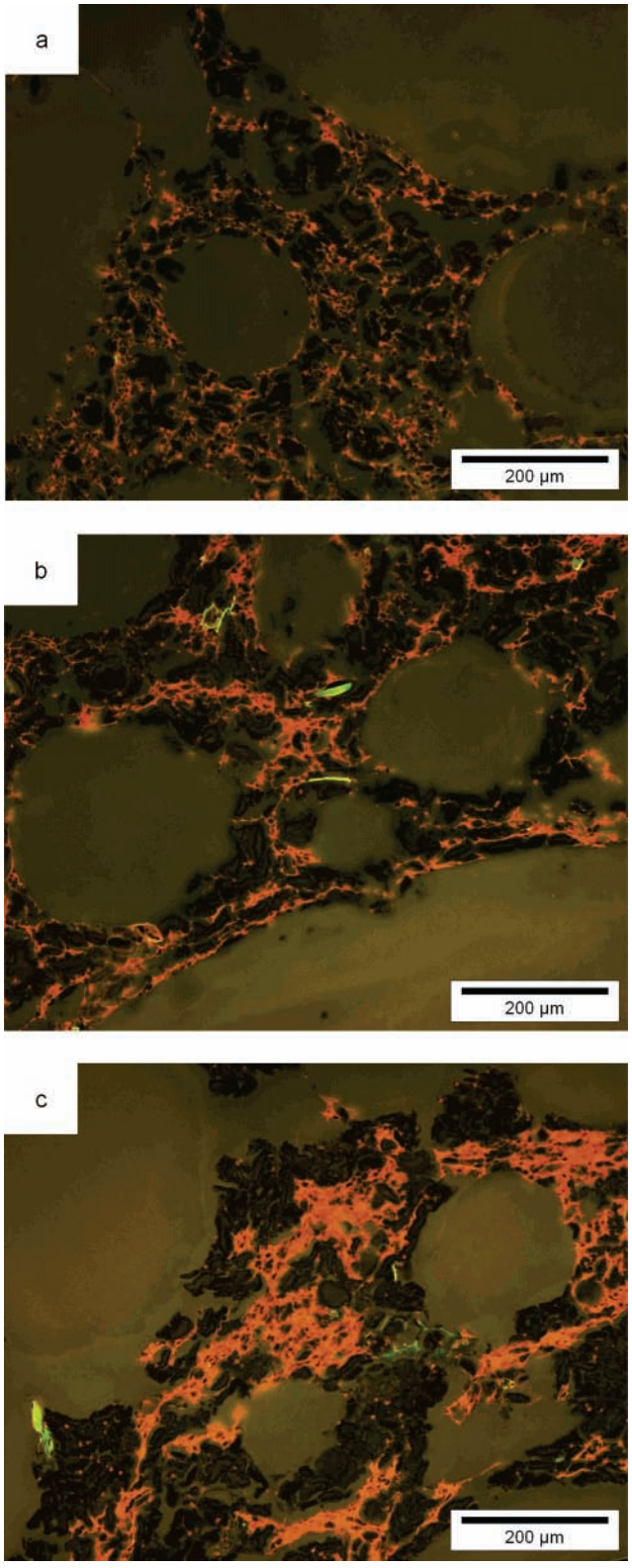

Figure 7. Micrographs of (**a**) the control bread, (**b**) bread containing 5.7 nkat/g TGase, and (**c**) bread containing 11.4 nkat TGase/g flour baked from organic flour at 10% of farinogram absorption. The protein has been stained orange by the acid fuchsin.

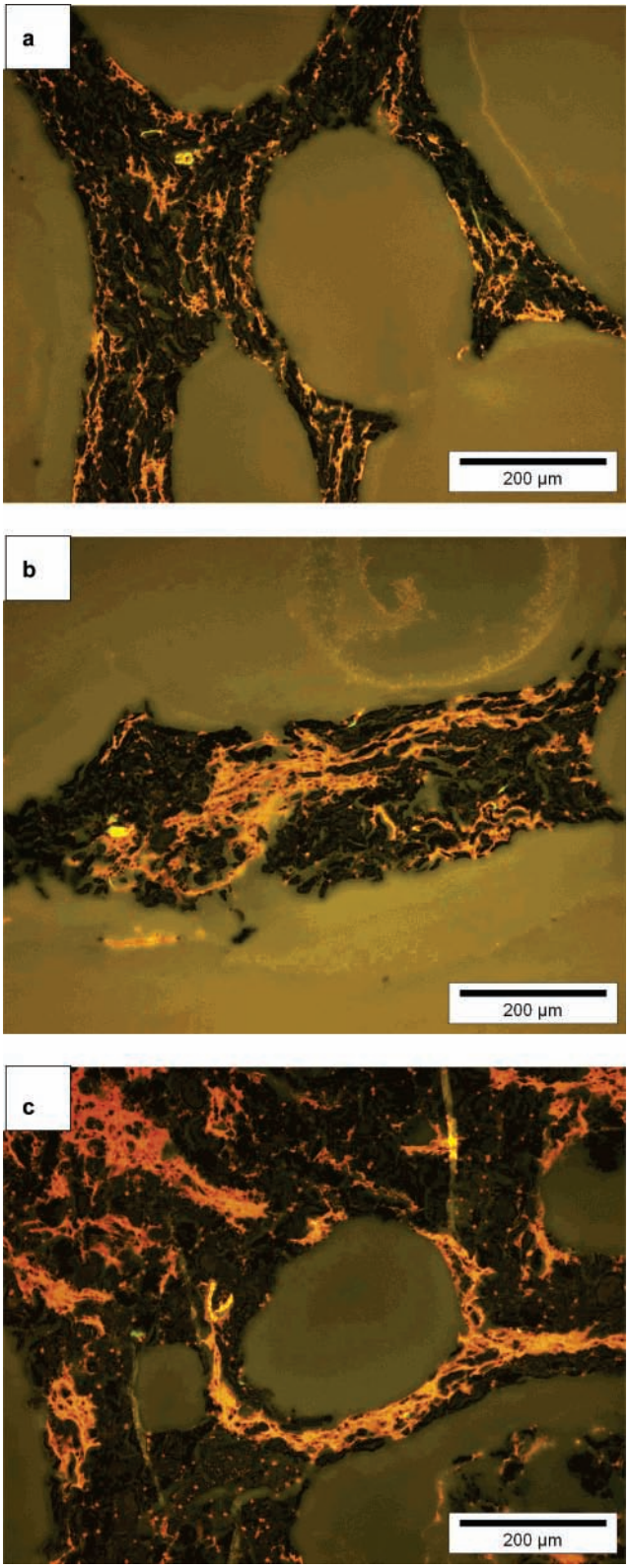

Figure 8. Micrographs of (**a**) control bread, (**b**) bread containing TGase at a activity level of 5.7 nkat/g, and (**c**) bread containing 11.4 nkat TGase/g flour baked from normal flour at 10% of farinogram absorption. The protein has been stained as in the previous figure.

breads baked with conventional flour corresponded to the point defined by water, 66.5−67% (f.w.), and TGase, 0−0.3% (f.w), yielding a specific volume = 3.9 cm^3/g. The corresponding optima (4.0 cm^3/g) for breads baked with organic flour were attained by adding 64.5−65% water (f.w.) and 0.4−0.6% TGase (f.w.) to the breads. TGase at 0.2% dosage increased the water absorption of the dough from 58 to 62%. GOX, which also cross-links glutenins in the dough, increases the dryness of the dough (*5*). If TGase is added to the dough without increasing the water content, the volume of the bread is reduced. The results shown in **Figures 2** and **3a**,**b** indicated that the TGase-treated

dough became less extensible very rapidly after mixing, which would further result in a lower dough and loaf volume. Studies with other cross-linking enzymes, GOX and POX, have shown that the best baking results were obtained by combining cross-linking enzymes with xylanases (*5*). With these enzymes in combination with xylanases, the stickiness was decreased and the handling properties improved.

Microstructure of Breads. The protein matrix in breads has been stained orange. The control bread baked from organic flour clearly had a looser protein network structure (**Figure 7a**) than the corresponding bread baked from normal flour (**Figure 8a**). The micrographs of breads baked from either organic (**Figure 7 a−c**) or normal (**Figure 8a−c**) flour showed that TGase promoted either the formation of thicker fibers or the interaction of fibers. The gluten network appears more developed in the presence of TGase. Glutenin molecules observed with the microscope showed orientated macrofibrils (*22*). Evans et al. (*23*) reported that doughs containing potassium bromate formed thicker fibers upon stretching. Another obvious effect was that the protein network was unevenly distributed especially at the highest dosage of TGase. One reason for the accumulation of the protein network due to TGase could be that during the expansion of dough, the strong protein fibers are not extended as much as they are in the control dough and are pushed together. Most of these protein-rich areas are located around air bubbles (**Figure 8c**).

To conclude, TGase makes the dough more rigid and less extensible quite quickly after mixing. TGase retarded the growth of air bubbles during fermentation. Microstructural studies indicated that TGase promoted gluten development but at the highest dosages caused uneven distribution of protein in the bread. The loaf volume could only be increased, when additional water was used. TGase had the potential to improve the baking quality of the weak flours.

ABBREVIATIONS USED

FA, ferulic acid; HMW, high molecular weight; GOX, glucose oxidase; LMW, low molecular weight; POX, peroxides; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TGase, transglutaminase.

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