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Elucidating the Mechanism of Laccase and Tyrosinase in Wheat Bread Making

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Cross-linking enzymes generate covalent bonds in and between food biopolymers. These enzymes are interesting tools for tailoring dough and bread structures, as the characteristics of the biopolymers significantly determine the viscoelastic and fracture properties of dough and bread. In this study, the influence of oxidative cross-linking enzymes, tyrosinase from the filamentous fungus *Trichoderma reesei* and laccase from the white rot fungus *Trametes hirsuta*, on dough and bread were examined. Oxidation of low molecular weight phenolic model compounds of flour, cross-linking of gluten proteins, dough rheology, and bread making were characterized during or after the enzymatic treatments. In the dough and bread experiments, laccase and tyrosinase were also studied in combination with xylanase. Of the model compounds tyrosine, *p*-coumaric acid, caffeic acid, ferulic acid, and Gly-Leu-Tyr tripeptide, tyrosinase oxidized all except ferulic acid. Laccase was able to oxidize each of the studied compounds. The phenolic acids were notably better substrates for laccase than L-tyrosine. When the ability of the enzymes to cross-link isolated gliadin and glutenin proteins was studied by the SDS−PAGE analysis, tyrosinase was found to cross-link the gliadin proteins effectively, whereas polymerization of the gliadins by laccase was observed only when a high enzyme dosage and prolonged incubation were used. Examination of large deformation rheology of dough showed that both laccase and tyrosinase made doughs harder and less extensible, and the effects increased as a function of the enzyme dosage. In bread making, interestingly, the pore size of the breads baked with tyrosinase turned out to be remarkably larger and more irregular when compared to that of the other breads. Nevertheless, both of the oxidative enzymes were found to soften the bread crumb and increase the volume of breads, and the best results were achieved in combination with xylanase.

KEYWORDS: Laccase; tyrosinase; cross-linking; wheat; dough; bread

INTRODUCTION

White wheat flour consists mainly of the starchy endosperm, which is separated from the bran and germ during milling. Generally wheat flour is composed of carbohydrates, 70−80%, proteins, 8−18%, lipids, 1.5−2.5%, and nonstarch polysaccharides, 2−3% (*1*, *2*). The most abundant component of flour is starch. In dough mixing starch granules become thoroughly embedded in the gluten network, and during baking, gelatinization of starch is essential for the development of a porous and elastic bread structure (*3*−*6*). Wheat proteins, albumins, globulins, and the gluten proteins, gliadins and glutenins, are considered as the most important components in flour, determining the bread quality with respect to the crumb structure and loaf volume. Gliadins and glutenins account approximately for 80% of the wheat proteins, and their viscoelastic nature is the backbone for the formation of a protein network during dough mixing (*7*, *8*). Of the wheat lipids, polar lipids have been reported to be advantageous to the bread loaf volume, whereas nonpolar lipids have been found to be mainly detrimental (*9*, *10*). Nonstarch polysaccharides of wheat flour are mostly built up of arabinoxylan (AX). AX is made up of linear β-1,4-linked xyloses substituted with arabinose residues on C-3 or on both C-2 and C-3 positions (*11*). Some of the arabinoses are esterified with phenolic acids, mainly ferulic acid (*11*). AX is also known to play an important role in the structure formation of the gluten network and dough. Water-unextractable AX has been reported to have a largely negative effect on the gluten yield, gluten composition, and rheological properties, whereas water-extractable AX is shown to have beneficial effects in bread making (*2*, *13*). Enzymatic hydrolysis of AX by xylanases (EC 3.2.1.8) causes water redistribution from AX to the gluten and starch phase, making the dough slacker, softer, and more viscous (*13*−*15*). When used at an appropriate level, xylanases have positive effects in bread making, such as improved dough extensibility, bread volume, and bread texture (*15*−*17*).

Texture plays a major role in the quality of bread. Textural properties are largely determined by the amount and balance of weak and strong physical linkages, hydrogen, hydrophobic, electrostatic, and covalent bonds, present in bread. Cross-linking enzymes are attractive tools to generate covalent bonds in the food matrix with subsequent changes in the textural properties

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Scheme 1

Laccase: 4 substrates (reduced) + O_2 → 4 substrates (oxidized) + 2 H_2O

Tyrosinase: 1 substrate (reduced) + O_2 → 1 substrate (oxidized) + H_2O (monophenolase)

2 substrates (reduced) + O_2 → 2 substrates (oxidized) + 2 H_2O (diphenolase)

of the final product. The impacts of enzymatic cross-linking have been elucidated in several cereal applications, including improving the dough handling properties, textural properties, elasticity, water holding capacity, firmness, and heat stability of cereal foods, especially in frozen dough baking (*18*−*24*). Currently, transglutaminase enzyme (EC 2.3.2.13), which is able to cross-link proteins via catalyzing acyl-transfer reactions between a γ-carboxyamine group of a peptide-bound glutamyl residue and an ϵ-amino group of a lysyl residue, is the commercially available cross-linking enzyme for cereal applications. Oxidative enzymes have also shown potentiality to cross-link food biopolymers. The oxidation can proceed in proteins and carbohydrates via phenolic moieties present in the polymers or through certain amino acid residues of proteins. The mechanism of oxidative enzymes in cereals is complicated as the enzymes may induce linkages in/between polysaccharides, in/between proteins, and between polysaccharides and proteins. Laccase (EC 1.10.3.2), tyrosinase (EC 1.14.18.1), and peroxidase (EC 1.11.1.7) directly, and also glucose oxidase (EC 1.1.3.4) indirectly via H_2O_2 production, are able to catalyze oxidation of phenolic compounds present in both proteins and polysaccharides. Glucose oxidase is hypothesized to participate in both protein network strengthening, especially via disulfide bonding, and oxidative gelation of AX (*18*, *25*). Peroxidase is proposed to promote cross-linking of the protein network and AX separately in dough (*19*, *21*), whereas laccase is reported to cross-link mainly AX in doughs via AX-esterified ferulic acid (*19*, *20*, *22*, *25*). Of the amino acids, tyrosine and cysteine are directly oxidized by laccase (*26*) and, naturally, tyrosine by tyrosinase. Lysyl, tyrosyl, cysteinyl, and histidinyl moieties are reported to react further with tyrosinase-oxidized tyrosine residues (*27*−*29*). Despite the fact that laccase has also been found to catalyze peptide polymerization (*26*), and to form cross-links in certain proteins (*30*, *31*), no evidence for the laccase-induced protein cross-linking in cereals has been reported so far. Piber and Koehler (*32*) have detected covalent cross-linking between feruloylated AX and tyrosyl residues of protein in wheat and rye, apparently caused by endogenous oxidative enzymes in grains. It has also been reported that FA and feruloylated AX can be linked to tyrosine-containing peptides and casein proteins by exogenous peroxidase (*33*, *34*).

Tyrosinases and laccases are copper-containing proteins. Enzymatic catalyses are based on an oxidative mechanism in which molecular oxygen is a terminal electron acceptor. Tyrosinase enzymes contain in their active site two type 3 copper atoms, which shuttle electrons from the substrate to the oxygen. Tyrosinases are monooxygenases and bifunctional enzymes which catalyze *ortho*-hydroxylation of monophenols and subsequent oxidation of *o*-diphenols to quinones (**Scheme 1**). Quinones can further react nonenzymatically to produce mixed melanins and heterogeneous polymers (*35*). Laccase enzymes contain four copper atoms in their active site. Laccases oxidize various phenolic compounds, e.g., diphenols, polyphenols, different substituted phenols, diamines, aromatic amines and thiols, and even some inorganic compounds such as iodine (*36*). Laccases oxidize their substrates with a one-electron removal mechanism, producing radicals, and the subsequent cross-linking of polymers is based on the high reactivity of the radicals. The free radicals can further undergo nonenzymatic reactions, including polymerization, hydration, and disproportionation (*36*).

In this study, the reactivity of laccase and tyrosinase in wheat flour dough and bread was examined. The aim was to elucidate how these enzymes affect the dough and bread and which of the flour components are mostly influenced by the enzymes. The oxidative enzymes were also studied in combination with xylanase.

MATERIALS AND METHODS

Enzymes. Laccase was produced by the white rot fungus *Trametes hirsuta* (ThL), purified by anion exchange chromatography and hydrophobic interaction chromatography (*37*). Laccase activity was determined according to Niku-Paavola et al. (*38*), using ABTS (2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) as the substrate. Specific activity for the purified ThL was 2500 nkat/mg. Tyrosinase (TrTYR2) was from the filamentous fungus *Trichoderma reesei*, overexpressed, produced, and purified as described by Selinheimo et al. (*39*). The activity of TrTYR2 was measured according to Robb (*40*), using 15 mM L-dopa (3,4-dihydroxy-L-phenylalanine) as the substrate, giving a specific activity of 250 nkat/mg. *Thermomyces lanuginosus* xylanase, heterologously expressed in *Aspergillus oryzae*, trade name Pentopan Mono BG (Xyl), was from Novozymes A/S (Bagsværd, Denmark). The activity of Xyl was determined in a reductometric assay according to Bailey et al. (*41*) with birch glucuronoxylan as the substrate, using colorometric dinitrosalicylic acid reagent and (1→4)-β-oligoxylosides as standards. The xylanase activity of the preparation was 132 600 nkat/g of enzyme powder. The protein content of Pentopan Mono BG enzyme powder was 2.2 mg/g, determined with a BioRad DC protein assay kit (Bio-Rad, Richmond, CA) with bovine serum albumin as the standard. Depending on the experiment, the enzymes were dosed on the basis of either their protein content (μg) or their activity (nkat/g of substrate), according to the individual activity determination of each enzyme.

Raw Materials. Wheat flour was commercial baking flour (Paakari, Finland). Model compounds L-dopa, caffeic acid (CafA), L-tyrosine (L-Tyr), and *p*-coumaric acid (*p*-CA) were from Sigma, and ferulic acid (FA), Gly-Leu-Tyr tripeptide (GLY), and gliadin from wheat were from Fluka BioChemika. Glutenins were extracted as described by Autio et al. (*42*).

The protein content determination of flour was based on the Kjeldahl method (*43*).

The caffeic, ferulic, and *p*-coumaric acid contents in flour were determined by HPLC (Millipore Waters, column Hypersil BDS-C18, Agilent Technologies) according to Bartolomé et al. (*44*). The total alkali-extractable content of phenolic acids in flour was determined by saponifying the samples in 1 M NaOH at 20 °C for 16 h under N_2. The mixture was acidified to pH 3 with acetic acid and extracted five times with equal volumes of ethyl acetate. The extracts were evaporated to dryness in a rotary evaporator, and the residue was dissolved in methanol/water (50/50, v/v), filtered through a 0.45 μm filter (Millex-HA, Millipore), and analyzed by HPLC.

Oxygen Consumption Measurement. Enzymatic oxidation of model compounds (2 mM) L-Tyr, *p*-CA, CafA, and FA and GLY peptide was examined by following the oxygen concentration in the reaction mixture. The compounds were dissolved in 0.1 M sodium phosphate buffer (pH 6.5), and the activity of ThL and TrTYR2 on the substrates was determined by following the consumption of the cosubstrate oxygen with a single-channel oxygen meter (Precision Sensing GmbH, Regensburg, Germany) at 25 °C in a closed and fully filled vial (1.8 mL). For L-Tyr, *p*-CA, CaFA, and FA, reactions were

initiated by addition of 20 μg of ThL and TrTYR2 to the substrate solutions, whereas for GLY, enzyme dosages were 20 and 70 μg of ThL and 20 and 200 μg of TrTYR2. The maximum oxidation rate (mg L^{-1} s^{-1}) was calculated from the linear part of the oxygen consumption curve.

Analysis of the GLY Peptide Oxidation by Capillary Electrophoresis (CE). The concentration of the GLY peptide was 2 mM, enzyme dosages were 20 and 70 μg of ThL and 20 and 200 μg of TrTYR2, and CE was run after enzyme incubations of 1 h. The CE system was a P/ACE MDQ instrument from Beckman (Fullerton, CA). Separations were run for 20 min at 30 kV in 0.1 M phosphate buffer (pH 2.5) at 20 °C in a fused silica capillary (Composite Metal Services, The Chace, Hallow, Worchester, U.K.), 50 μm i.d. (55/65 cm long). The sample injection was done hydrodynamically by overpressure (34.5 kPa, 5 s). The separations were run with positive polarity at the inlet, and detection was done at 214 nm.

Protein Cross-Linking Assay with SDS−PAGE. The ability of the enzymes to cross-link cereal proteins, gliadins and glutenins, was studied by a SDS−PAGE analysis. The protein concentration was 1 mg/mL (dissolved in 0.1 M sodium phosphate buffer, pH 6.5), the incubation time was 15−255 min (samples were taken from the reaction solution at 30 min intervals), and the enzyme dosages were 0.1, 0.3, 1, 3, and 10 (with ThL also 15 and 150) nkat/mg of protein. The lowest doses were 10 times that used in dough treatment as flour contains only about 10% gluten (*45*). SDS−PAGE (12% Tris−HCl Ready Gel, Bio-Rad) assay was performed according to Laemmli et al. (*46*) using prestained SDS−PAGE standards (broad range, catalog no. 161-0318, Bio-Rad) and Coomassie Brilliant Blue (R350, Pharmacia) for staining the proteins.

Rheological Tests. Large deformation rheological tests were performed by uniaxial extension measurements at ambient temperature. Flour dough was prepared with a Mixograph (National Manufacturing Co., Lincoln, NE) by mixing 12 g of flour for 3.5 min with 7.08 mL (59%) of distilled water. ThL and TrTYR2 dosages of 5, 10, and 30 nkat/g of flour were added to the water phase just before it was mixed with the flours. Xylanase powder with dosages of 50 and 500 nkat/g of flour was mixed into the flour. TrTYR2 was also tested in combination with xylanase as follows: 12 g of flour was mixed with 6 mL (50%) of distilled water with a tyrosinase dosage of 5 nkat/g of flour and xylanase dosages of 125 and 875 nkat/g of flour. All dough samples were characterized using the Kieffer dough and gluten extensibility rig fitted onto a TA.XT2 texture analyzer (Stable Micro Systems, Ltd., U.K.) equipped with a 5 kg load cell. Tests were performed according to Kieffer et al. (*47*) with some modifications with respect to resting times and temperatures. The dough was moulded in the press immediately after mixing and kept pressed for 15−45 min to allow stress relaxation. Measurements of three parallel doughs were performed.

Microscopic Examination of Enzyme-Treated Doughs. For the microscopical examination, pieces (i.d. $\approx$ 0.5 cm) of enzymatically treated doughs were first 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 2 μm thick in a Leica rotary microtome HM 355 (Leica, Heidelberg, Germany) and transferred onto glass slides. Prior to the examination on the microscope, three different stainings were carried out. With 0.1% Acid Fuchsin (Gurr, BDH Chemicals Ltd., Poole, England) and 0.01% Calcoflour (Fluorescent Brightener 28, Aldrich, Germany), the cell walls were dyed blue and the protein was dyed orange and light gray; with Light Green (Gurr, BDH Chemicals Ltd.) and Lugol's iodine solution (I_2, 0.33%, w/v; KI, 0.67%, w/v) the starch was dyed dark blue and the protein green. In addition, a staining with protein-sensitive Xylidine Ponceau, 0.2% (Gurr, BDH Chemicals Ltd.), was performed. The stained samples were examined with an Olympus BX-50 microscope (Tokyo, Japan) connected to a SensiCam PCO CCD camera (Hamamatsu Photonics K.K., Hamamatsu-City, Japan) with an Analysis 3.0 image analysis program (Soft Imaging System, Münster, Germany).

Bread Making. The effects of TrTYR2 and ThL alone and in combination with Xyl on the wheat bread structure were studied by measuring the specific volume and hardness of the breads and observing the bread crumb structure (pore size) by visual examination. The specific volume of the bread was determined by measuring the bread weight (g) and volume (mL). The hardness of the bread crumbs 2 and 72 h after baking was analyzed by texture profile analysis (TPA) (TA.XT2 texture analyzer, Stable Microsystems Ltd., U.K.). Two different bread-making scales, doughs of 20 and 200 g, were studied, and both bread-making procedures were performed twice. Enzyme dosages of 5 and 10 nkat/g of flour of ThL and TrTYR2 and a dosage of 50 nkat/g of flour of Xyl were used. For 20 g scale bread making, wheat flour (100 g), 2 g of baker's yeast, 2 g of sugar, 1.5 g of salt, and 2 g of margarine were mixed with 67 mL of water in a HOBART mixer for 5 min at 22 °C. The dough was removed from the mixer, hand-moulded, and allowed to rest for 20 min at 28 °C in a 75% water-saturated atmosphere. Subsequently, the dough was divided into four 20 g pieces, shaped on a dough rounder (Brabender Extensigraph, Germany), and sheeted on a pasta machine; the resulting oval dough piece was bent over twice and reshaped manually. The resulting spherical dough piece was proofed for 35 min at 30 °C (75% water-saturated atmosphere) and baked for 13 min at 210 °C; in the beginning of baking, the oven was steamed for 15 s. For 200 g scale bread making, wheat flour (1200 g), 24 g of baker's yeast, 24 g of sugar, 11 g of salt, and 24 g of margarine were mixed with 704 mL of water. The doughs were kneaded with a spiral kneader (Diosna SP 12 F, Dierks & Sohne, GmbH, Osnabruck, Germany) for 2 min at a low speed (100 rpm), followed by 5 min at high speed (200 rpm). After the intermediate proof (20 min, 28 °C, 75% relative humidity), the dough was divided into nine 200 g pieces and moulded by hand. The moulded dough pieces were placed in aluminum shells and proofed at 37 °C and 80% relative humidity for 55 min. The loaves were baked for 18 min at 210 °C (Rack Oven 9000, Sveba Dahlen AB, Sweden). Steam was added for 20 s during the initial baking phase.

Table 1. Enzymatic Oxidation of Model Substrates (2 mM), *p*-Coumaric Acid, Caffeic Acid, Ferulic Acid, L-Tyrosine, and GLY, by 20 μg of TrTYR2 and ThL Determined by Oxygen Consumption Measurement

substrate	oxygen consumption rate (mg L^{-1} s^{-1})		rate in relation to caffeic acid (%)		content in flour (μM)
	TrTYR2	ThL	TrTYR2	ThL	
p-coumaric acid	0.030	0.015	43	79	0.001[a]
caffeic acid	0.070	0.019	100	100	0.002[a]
ferulic acid	0.000	0.019	0	100	0.5[a]
L-tyrosine	0.008	0.002	11	11	25[b]
GLY	0.016	0.004	23	21	

[a] Determined by HPLC. [b] Calculated according to the L-tyrosine content of proteins, 3% (*8*), and protein content of flour, 13.7%, as determined by the Kjeldahl method (*43*).

RESULTS

Analysis of the Flour Composition. The potential phenolic sites for enzymatic cross-linking in flour were analyzed. The protein content of wheat flour was 13.7%, and as the tyrosine (L-Tyr) content of wheat proteins is around 3% (*8*), it can be approximated that the maximum L-Tyr content of flour was 0.0045 g/g of flour, i.e., 25 μM. The amounts of phenolic acids FA, CafA, and *p*-CA in flour were 96.8, 0.4, and 0.2 μg/g, corresponding to 0.5, 0.002, and 0.001 μM, respectively. Therefore, the L-Tyr content of wheat flour is roughly at least 50 times higher than the content of phenolic acids.

Oxidation of the Model Compounds. The oxidation efficiency of L-Tyr, FA, CafA, and *p*-CA by the oxidative enzymes was studied by measuring the oxygen consumption during enzymatic reactions (**Table 1**). L-Tyr, CafA, and *p*-CA were oxidized by TrTYR2, whereas TrTYR2 could not oxidize FA.

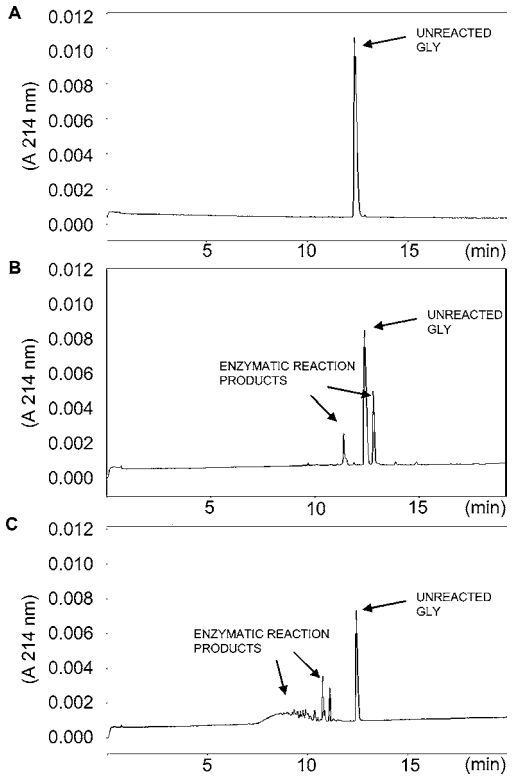

Figure 1. Product patterns from oxidation of GLY (2 mM) by ThL and TrTYR2 after 1 h of incubation, analyzed by capillary electrophoresis (running at 30 kV, 20 min in 0.1 M phosphate buffer, pH 2.5): (**A**) GLY reference, (**B**) GLY with 200 μg of TrTYR2, (**C**) GLY with 70 μg of ThL.

When calculated in relation to L-Tyr, the oxidation rate of CafA and *p*-CA by TrTYR2 was 8.8 and 3.8 times higher, respectively. ThL was able to oxidize all tested model compounds. Nevertheless, when compared to L-Tyr, the oxidation of FA, CafA, and *p*-CA by ThL was 7.5, 9.5, and 9.5 times faster, respectively. When the oxygen consumption rates between the enzymes are compared, it should be remembered, that according to the stoichiometry of the laccase reaction, oxidation of one phenolic molecule requires only one-fourth of an O_2 molecule, whereas in the case of tyrosinase one monophenolic molecule needs one oxygen molecule and one diphenolic molecule needs half an oxygen molecule in the reaction to form a quinone (**Scheme 1**). Consequently, when compared to tyrosinase, laccase consumes 2 and 4 times less oxygen to oxidize mono- and diphenols, respectively.

Both enzymes were able to oxidize peptide-bound tyrosine (GLY), and even faster than the amino acid L-Tyr (**Table 1**). Product formation from the oxidation of the tripeptide was followed with the CE analysis (**Figure 1**). The product patterns of GLY oxidation were notably different between the enzymes. TrTYR2-mediated catalysis gave a less diverged product pattern for GLY, when compared to the ThL-mediated oxidation of the tripeptide. The increase in enzyme dosage did not significantly change the product patterns; only the ratio of substrate to product amount decreased as expected (data not shown). A clear reference for the difference in the mode of action of the enzymes was also the observed color formation in the reaction solutions of GLY. Both enzymes were able to oxidize the peptide; however, after the reaction with TrTYR2 the color of the GLY solution shifted to red, whereas the solution of ThL-treated GLY remained colorless.

Effect of the Enzymes on the Molecular Weight of Gliadins. The ability of the enzymes to cross-link isolated gliadin proteins was studied by the SDS−PAGE analysis (**Figure 2**). TrTYR2 was able to cross-link the gliadins effectively, as visualized by the decrease in the intensity of the gliadin-specific subunits and by the formation of higher molecular weight products in the gel already after 15 min of reaction time (**Figure 2A**, lanes 3−6). The efficiency of cross-linking increased as a function of tyrosinase dosage and reaction time (data not shown). After the 15 min reaction time, the effect of ThL on gliadins was not as visible as the effect of TrTYR2; barely few higher molecular weight compounds were formed, and only with a high ThL dosage (10 nkat/mg of gliadin) (faintly

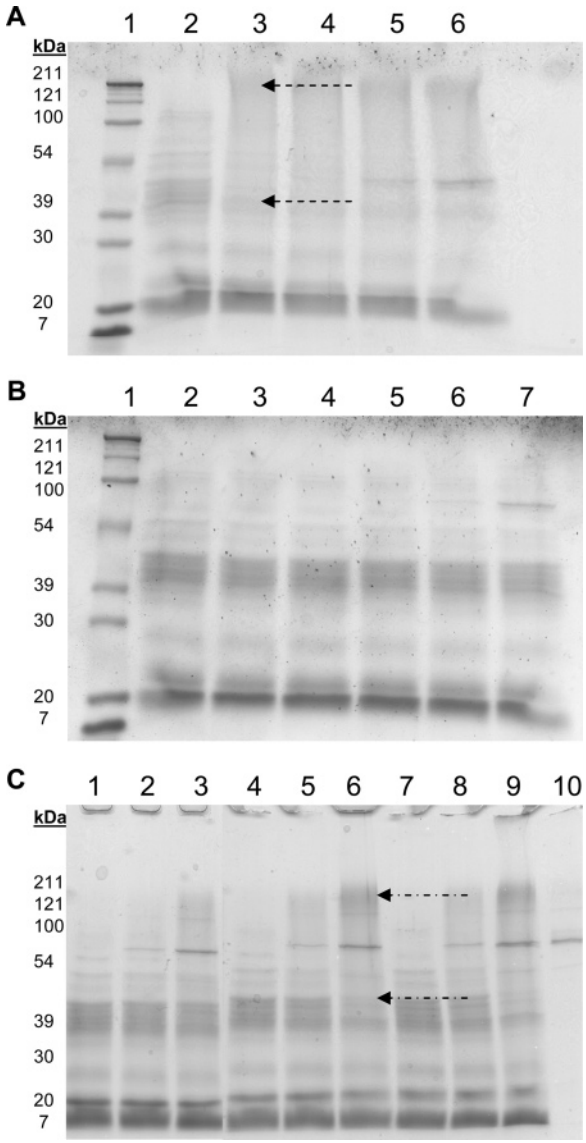

Figure 2. Cross-linking of gliadin proteins by ThL and TrTYR2, determined on an SDS–PAGE gel. Enzyme dosages (nkat) are per milligram of gliadin. (**A**) Gliadin with TrTYR2 (15 min incubation). Lanes: (1) molecular weight marker, (2) reference, (3) TrTYR2, 0.1 nkat, (4) TrTYR2, 0.3 nkat, (5) TrTYR2, 1 nkat, (6) TrTYR2, 3 nkat. (**B**) Gliadin with ThL (15 min incubation). Lanes: (1) molecular weight marker, (2) reference, (3) ThL, 0.1 nkat, (4) ThL, 0.3 nkat, (5) ThL, 1 nkat, (6) ThL, 3 nkat, (7) ThL, 10 nkat. (**C**) gliadin with ThL (30, 45, and 75 min incubations). Lanes: (1) reference (30 min), (2) ThL, 15 nkat (30 min), (3) ThL, 150 nkat (30 min), (4) reference (45 min), (5) ThL, 15 nkat (45 min), (6) ThL, 150 nkat (45 min), (7) reference (75 min), (8) ThL, 15 nkat (75 min), (9) ThL, 150 nkat (75 min), (10) enzyme control, ThL, 150 nkat (without gliadin). Major changes are illustrated with arrows.

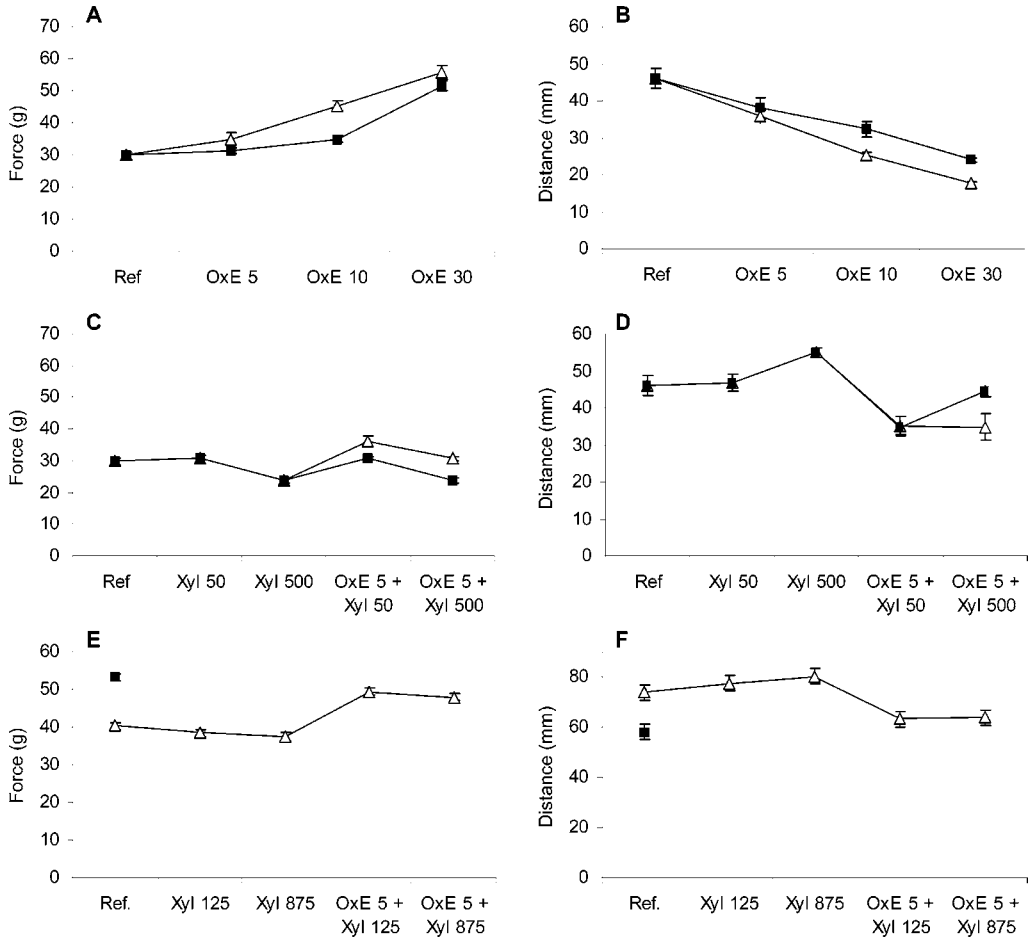

Figure 3. Effect of oxidative enzymes (OxEs), ThL and TrTYR2, as a function of dosage on the dough rheology in combination with Xyl: (**A**, **C**, **E**) dough strength (force/g), (**B**, **D**, **F**) dough extensibility (distance/mm). Symbols in (**A**)–(**D**): ThL and Xyl with ThL (■), TrTYR2 and Xyl with TrTYR2 (△). Symbols in (**E**) and (**F**): TrTYR2, 5 nkat/g of flour (■), Xyl with TrTYR2 (OxE) (△). Numbers on the *x* axes correspond to enzyme doses as nkat/g of flour.

seen in **Figure 2B**, lane 7). However, when ThL was incubated longer with the gliadins, clear cross-linking of the proteins was observed, especially with the higher ThL dosages (15 and 150 nkat/mg of gliadins) (**Figure 2C**, lanes 3, 6, and 9).

Effect of the Enzymes on Dough Rheology. The effect of the oxidative enzymes alone, and in combination with xylanase, on the rheological properties of wheat dough was studied by a large deformation rheology measurement, the Kieffer test, which enables the detection of covalent cross-link formation in dough. The measurement hook extends the dough string until its elastic limit is exceeded and it ruptures; thereby, the maximum resistance (a peak force) and extensibility (distance of the hook from the start point) of the dough string are recorded. Both ThL and TrTYR2 made doughs harder and more inflexible, as indicated by an increase in the strength (**Figure 3A**) and a decrease in the extensibility (**Figure 3B**). As expected, xylanase made the dough more extensible and softened the dough (**Figure 3C,D**). The effects were increased as a function of enzyme dosage. Considering the combined treatments of the oxidative enzymes with Xyl, in the ThL−Xyl treatments, the influence of ThL on the dough structure seemed to be predominant at low xylanase dosages. When xylanase was added at high dosage, the hardening effect of ThL on the dough was clearly decreased, and the influence of xylanase appeared to be stronger. In the TrTYR2−Xyl treatments, softening of the dough structure with increased xylanase dosage was not at all as evident as it was with the ThL−Xyl treatments, and the hardening effect by TrTYR2 seemed to dominate, especially when a lower water content (50%) in the dough was used (**Figure 3E,F**).

The impact of the enzymatic treatments on the different flour components, protein, cell wall (e.g., arabinoxylan), and starch, was also studied by microscopy with three different staining procedures (**Figure 4**). In **Figure 4**, in the slides 1, 2, and 3, proteins are dyed green, red, and red-brown, respectively. When the control dough (**Figure 4A**) was compared with the ThL- and TrTYR2-treated doughs (**Figure 4B,C**, respectively), the effect of the oxidative enzymes seemed to be fairly similar. The proteins of flour were predominantly affected by the enzymes, seen as a formation of protein-rich areas and large gluten macrofibrils, i.e., the green, red, and brown clusters in **Figure 4**, slides 1, 2, and 3, respectively. Interestingly, no clear differences were observed in the cell walls after the enzymatic treatments (**Figure 4**, cell walls dyed blue in slides 3). With the lower enzyme dosages of ThL and TrTYR2 (5 and 10 nkat/g of flour), no significant changes were observed in the microscopical analysis, as compared to the reference (data not shown).

Effect of the Enzymes in Bread Making. The influences of TrTYR2, ThL, and Xyl were elucidated in two different scale bread-making tests. The hardness of the breads 2 and 72 h after baking (**Figure 5**), the specific volume (mL/g) of the breads (**Table 2**), and the pore size of the crumb of the breads (**Figure 6**) were analyzed. The hardness of the breads was corrected for the specific volume (**Figure 5**). In the 20 g scale bread making, differences between the breads with respect to the hardness and

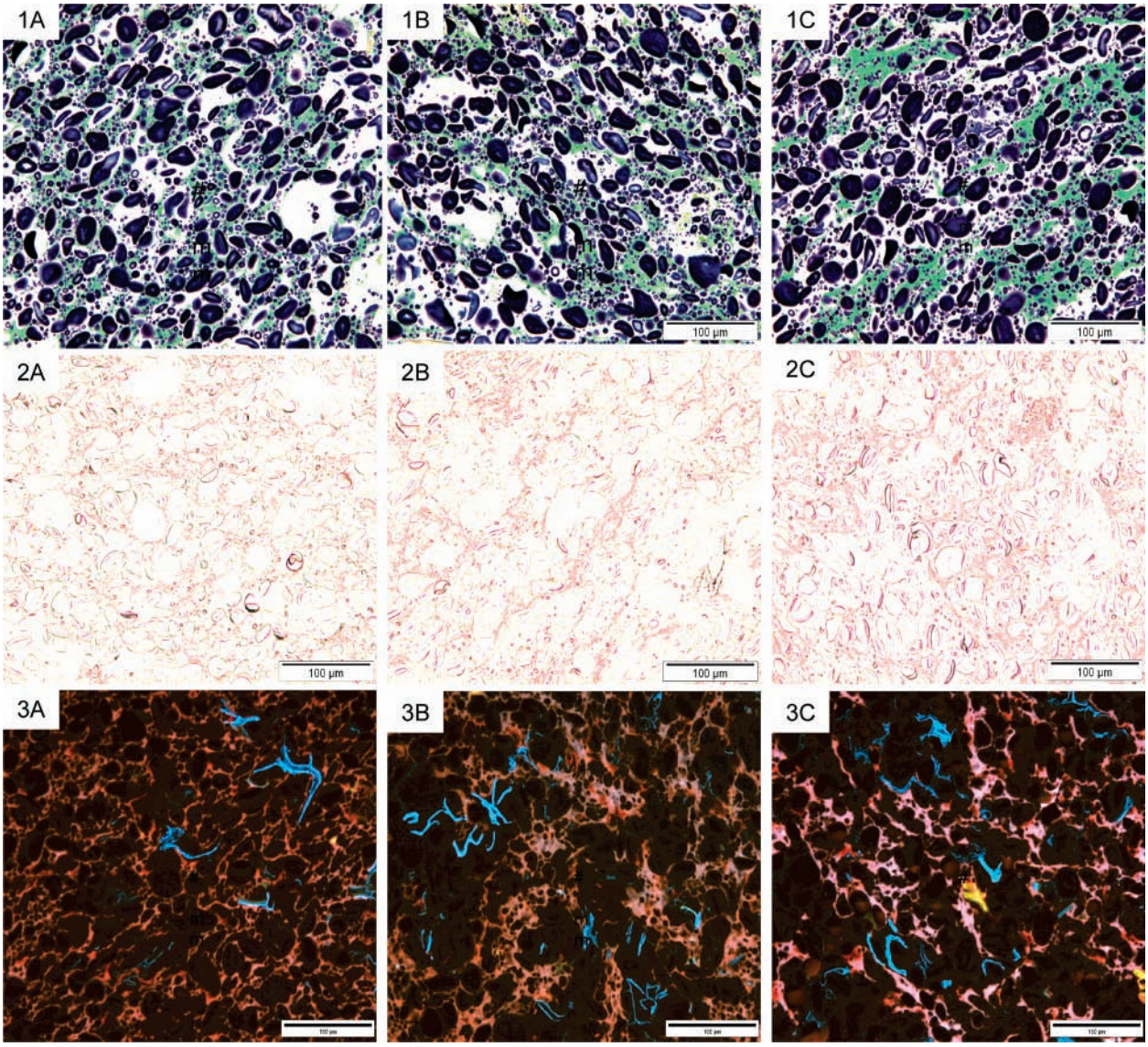

Figure 4. Microscopy of doughs treated with ThL and TrTYR2 (scale bar 100 μm). Stainings: (1) Light Green and Lugol's iodine solution, starch dyed dark blue and protein green, (2) Xylidine Ponceau, 0.2%, protein dyed red, (3) 0.1% Acid Fuchsin and 0.01% Calcoflour cell walls dyed blue and protein orange and/or light gray. Treatments: (**A**) reference, (**B**) ThL, 30 nkat/g of flour, (**C**) TrTYR2, 30 nkat/g of flour.

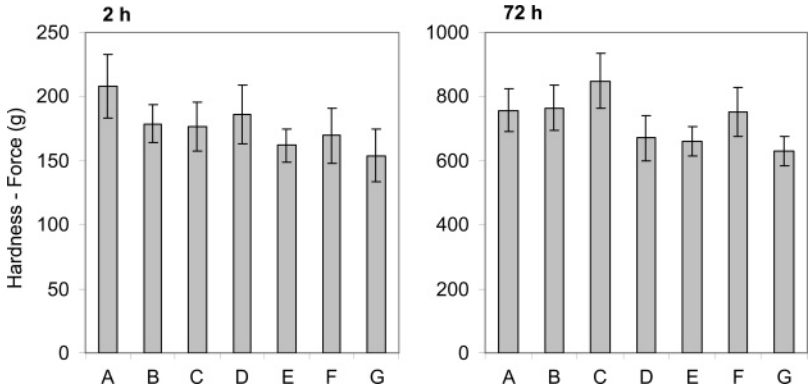

Figure 5. Effect of ThL, TrTYR2, and Xyl on the hardness (force/g) of wheat bread, measured 2 and 72 h after baking. Enzyme dosages (nkat) are per gram of flour. Column legends: (**A**) reference, (**B**) TrTYR2, 5 nkat, (**C**) TrTYR2, 10 nkat, (**D**) Xyl, 50 nkat, (**E**) TrTYR2, 5 nkat, with Xyl, 50 nkat, (**F**) ThL, 5 nkat, (**G**) ThL, 5 nkat, and Xyl, 50 nkat.

the specific volume of the breads treated with different enzymes proved to be statistically insignificant. The most probable reason for this is the high proportion of crust in the 20 g scale breads in relation to the bread crumb. According to the hardness measurements of breads from the 200 g scale bread making, all of the enzyme treatments softened the bread structure compared to that of the control bread (**Figure 5**). The softest breads were obtained after combined treatments: TrTYR2 with Xyl and ThL with Xyl. Furthermore, the Xyl and the combined treatments retained best the softness of the breads (for 72 h),

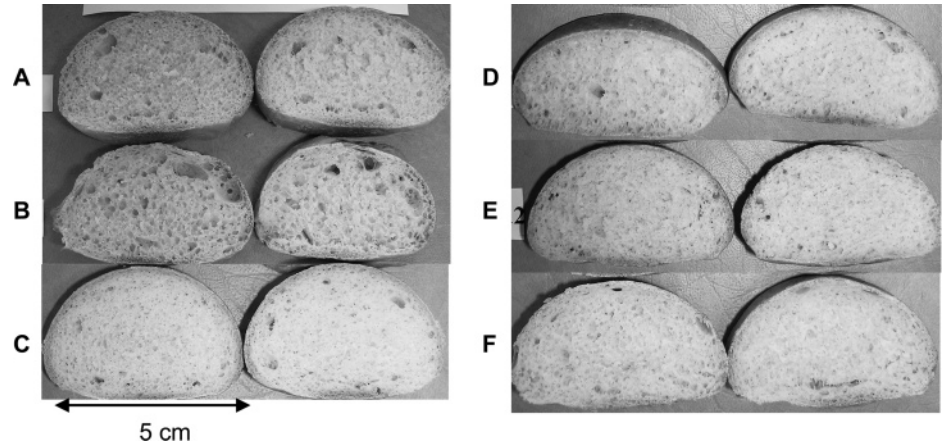

Figure 6. Visual examination of the effect of ThL, TrTYR2, and Xyl on the pore size of the crumb structure of 20 g scale breads. Enzyme dosages (nkat) are per gram of flour. Key: (**A**) reference, (**B**) TrTYR, 5 nkat, (**C**) ThL, 5 nkat, (**D**) Xyl, 50 nkat, (**E**) TrTYR, 5 nkat, with Xyl, 50 nkat, (**F**) ThL, 5 nkat, with Xyl, 50 nkat.

Table 2. Effect of TrTYR, ThL, and XYL on the Specific Volume (mL/g) of Wheat Breads (200 g Scale) with the Standard Deviation (std dev)[a]

treatment	specific vol of the breads (mL/g)	std dev
reference	3.29	0.07
TrTYR2, 5 nkat	3.60	0.08
TrTYR2, 10 nkat	3.76	0.06
Xyl, 50 nkat	3.73	0.07
TrTYR2, 5 nkat, + Xyl, 50 nkat	3.79	0.08
ThL, 5 nkat	3.73	0.08
ThL, 5 nkat, + Xyl, 50 nkat	3.77	0.06

[a] Enzyme dosages: TrTYR2, 5 and 10 nkat/g of flour; ThL, 5 nkat/g of flour; Xyl, 50 nkat/g of flour.

whereas the breads treated with ThL and TrTYR2 alone lost more of their softness during storage. Increasing the TrTYR2 dosage from 5 to 10 nkat/g of flour increased the bread hardness during storage. When compared to that of the control bread, the specific volume of the baked breads was slightly increased by an addition of all of the enzymes (**Table 2**). The largest bread volumes were observed after the combined treatments: TrTYR2 with Xyl and ThL with Xyl. In addition, although the softness of the bread was not increased by increasing the TrTYR2 dosage from 5 to 10 nkat/g of flour, the bread volume was slightly increased as a function of TrTYR2 dosage. Xyl alone also increased the volume and softness of the bread, but the doughs treated with Xyl alone were sticky and difficult to handle during the bread-making process. However, the machinability of the Xyl-treated doughs improved when ThL and TrTYR2 were added in combination with Xyl.

In the 20 g scale bread making, the pore size of bread crumbs baked with TrTYR2 was observed to be noticeably larger and more irregular when compared to that of the control and the Xyl- or the ThL-treated breads (**Figure 6**). However, an addition of xylanase with TrTYR2 changed the bread crumb's pore size to more regular and smaller. With larger 200 g scale breads, this tyrosinase-related phenomenon of irregular crumb structure was found to be decreased. However, with both the ThL- and TrTYR2-treated breads the crust on top of the bread was coarser when compared to that of the control and Xyl-treated breads. During the bread-making process, especially during proofing and baking, some unusual bubble formation was observed with TrTYR2- and ThL-treated breads. Probably an abnormal gas bubble formation and rupturing of the bubbles during baking caused the coarse crust structure detected in the 200 g scale bread making, whereas in the 20 g scale bread making, in which the crust was particularly stronger, the bubbles were not able to diffuse through the crust, but stayed in the bread crumb, resulting in an irregular pore size of the crumb as seen in the TrTYR2 treatments.

DISCUSSION

The tyrosinase and the laccase of fungal origin, TrTYR2 and ThL, could oxidize phenolic compounds present both in wheat proteins, i.e., L-Tyr, and in AX, i.e., CafA, *p*-CA, and FA. ThL could oxidize all of the tested compounds, whereas TrTYR2 oxidized all with the exception of FA. A similar substrate specificity for TrTYR2 has been previously reported by Selinheimo et al. (*39*), and the inactivity toward ferulic acid has been concluded to be due to a methoxy side group next to the phenolic hydroxyl group of FA, preventing the hydroxylation of the substrate prior to the oxidation. Oxidation of various phenolic acids by ThL has been widely reported (*36*, *37*), but L-Tyr was a rather poor substrate for ThL as previously described also by Mattinen et al. (*26*). It is noteworthy that, although the oxidation rate of L-Tyr by ThL and TrTYR2 was clearly slower than that of phenolic acids, the total content of FA, CafA, and *p*-CA in wheat flour is approximately 50 times lower than the content of tyrosine. In the TrTYR2-catalyzed reactions, the low phenolic acid/L-Tyr ratio becomes even more evident. Tyrosinase is not able to oxidize FA, the major phenolic acid component of arabinoxylan, which makes the ratio of reactive sites of AX-bound phenolic acids to protein-bound L-Tyr even as low as 1/8000. Naturally, it is not only the oxidation efficiency of the model compounds determining the effects of the enzymes in the dough and bread, but also the amount, distribution, and availability of these phenolic moieties for the enzymatic oxidation in the polymers.

Both ThL and TrTYR2 were able to oxidize the model peptide GLY. However, according to CE analysis, the reaction product patterns were found to be different. The differences in the product formation are most presumably related to the different mechanisms of the enzymes; the tyrosinase-catalyzed cross-linking is based on the quinone formation and their further nonenzymatic reactions, whereas the laccase-catalyzed cross-linking is based on the generation of free radicals.

Tyrosinase seemed to be clearly more effective in the cross-linking the gliadin proteins than laccase. When the corresponding cross-linking assay was performed with wheat glutenins, similar results were achieved, effective cross-linking by TrTYR2 and faint cross-linking by ThL (data on glutenins not shown). Despite the capability of laccase to oxidize the amino acid L-tyrosine, and the peptide bound tyrosine (GLY), cross-linking of proteins by laccase was only seen with high enzyme dosages and with longer incubation times. Previously, laccases have been

reported to cross-link proteins other than gliadins or glutenins (*30*, *31*); however, this is the first report where also the wheat gluten proteins were cross-linked by laccase.

Both ThL and TrTYR2 had a clear hardening effect on the wheat flour dough as measured by a dough rheology test; the dough extensibility was decreased and the strength increased. The effect of ThL is assumed to be based mainly on the cross-linking of AX via ferulic acid side groups, resulting in a strong AX network, and thereby also strengthening the gluten network (*19*, *20*, *22*). This hypothesis is also supported by the result where the higher xylanase dosage (500 nkat/g of flour) was added in combination with ThL, with a consequential decrease in the hardening effect of ThL on the dough. A similar xylanase-related softening phenomenon in the laccase-treated wheat doughs was observed previously (*49*). Apparently, with higher xylanase dosages AX is so fast and thoroughly hydrolyzed that ThL is not able to create the AX network. However, somewhat contradictory results were observed when the enzymatically treated doughs were studied under a microscope. Although laccase is generally known to act on AX (*19*, *20*, *22*), according to the microscopic analysis, the flour proteins of the dough seemed to be affected by ThL and not the cell wall structures. However, only some cross-linking of gliadins by ThL was detected by the SDS−PAGE assay. Therefore, it is assumed that the cross-linked high-molecular-weight AX could act as a matrix reinforcer by increasing the effective concentration of gluten in the dough. On the other hand, formation of the FA radicals by enzymatic oxidation has been reported to increase the oxidation rate of the free SH groups (*22*). Thereby, the FA-derived radicals from the oxidation of AX-linked FA by ThL could also have influenced the natural disulfide (S−S) formation in gluten polymers, leading to the uncharacteristic gluten and dough structure formation. Furthermore, ThL oxidizing and radicalizing directly the tyrosyl residues of the gluten proteins, and thus assisting the protein aggregation in the dough, can not be excluded.

TrTYR2 was clearly more effective in cross-linking gluten proteins compared to ThL. In the microscopical examination, as well, the flour proteins were observed to be the most significantly affected by TrTYR2. Thus, the observed effects on the dough rheological properties are most probably due to the alterations in the gluten polymers of the flour. This conclusion is consistent with the dough rheology results obtained from the combined TrTYR2−xylanase experiment, where the effect of the xylanase dosage was remarkably weaker than what was found in the ThL−xylanase treatments. As TrTYR2 affects mainly the gluten proteins, hydrolysis of AX by xylanase is probably not so clearly detectable in the rheological properties of the dough.

Both ThL and TrTYR2 increased the softness of the bread crumb and bread volume, although the best results were achieved in combination with xylanase. No distinct explanation for the basis of this combinatory effect could be disclosed, and the interesting observation made in the bread-making tests about the irregular and large pore size by TrTYR2 treatment cannot be comprehensively explained either. A large and irregular pore size of the bread crumb has typically been observed when the formation of the gluten matrix is somehow disturbed, for instance, when water-insoluble cell wall material, such as AX, is included in the flour (*10*, *14*, *50*). Transglutaminase that is known to act only on the proteins in the dough is reported to cause a firm bread crumb with a small and regular pore size (*42*), which is opposite what was detected with tyrosinase. Tyrosinase produces reactive quinones, which can couple to thiol and amino groups and also to other quinones (*27*). Therefore, tyrosinase could catalyze, for instance, in proteins, the formation of dityrosine, tyrosine−cysteine, and tyrosine−lysine cross-links. Especially, the tyrosine−cysteine linkages could disturb the proper gluten structure formation, by hindering the intra- and interchain S−S conjugation of the gluten proteins. Thereby, the alterations in the gas-retaining ability of the gluten network could partly explain the irregular and uneven pore size of tyrosinase-treated breads. On the other hand, addition of Xyl with TrTYR2, interestingly, changed the pore size of the bread crumb to more regular and smaller, which also suggests AX to have been affected in the structure formation of the TrTYR2-treated breads, for instance, via oxidation of AX-bound *p*-CA and CafA. Furthermore, it has been hypothesized that AX can influence gluten indirectly by changing the water distribution in the dough and by also having direct interactions with gluten (*11*, *13*, *15*). Xyl by hydrolyzing AX could have diminished the possible tyrosinase-mediated association of AX with gluten, resulting in an improved formation of the gluten network.

Although laccase and tyrosinase were observed to act in the wheat dough and bread via different cross-linking mechanisms, their influence on the structural properties of the dough and bread seemed to be rather similar. Both laccase and tyrosinase increased the dough strength and improved the bread-making quality of white wheat flour breads, especially when used in combination with xylanase.

ABBREVIATIONS USED

L-Tyr, L-tyrosine; FA, ferulic acid; CafA, caffeic acid; *p*-CA, p-coumaric acid; GLY, Gly-Leu-Tyr peptide; AX, arabinoxylan; TrTYR2, tyrosinase from *Tri. reesei*; ThL, laccase from *Tra. hirsuta*; Xyl, Pentopan Mono BG xylanase; CE, capillary electrophoresis.

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