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# Effects of Tyrosinase and Laccase on Oat Proteins and Quality Parameters of Gluten-free Oat Breads

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**ABSTRACT:** Effects of a *Trichoderma reesei* tyrosinase (TYR) and a *Trametes hirsuta* laccase (LAC) on breadmaking performance of gluten-free oat flour were investigated by SDS-PAGE analysis of oat protein fractions, large deformation rheology, and microscopy of the doughs, as well as on the basis of specific volume and firmness of the gluten-free breads. TYR induced the formation of higher molecular weight proteins in the SDS-PAGE assay. Microscopical analysis showed more intensive protein aggregation in the TYR-treated dough than in the dough without TYR. TYR also increased the firmness of the dough, which was assumed to be because of the cross-linking of oat globulins. LAC did not affect the oat globulins. TYR alone, or together with a commercial *Thermomyces lanuginosus* xylanase (XYL), increased significantly the specific volume of the gluten-free oat bread. A combination of TYR and XYL also increased the softness of the bread, whereas a combination of LAC and XYL improved the specific volume but did not affect the softness of oat bread. The results thus indicate that cross-linking of oat globulins by TYR, especially with the addition of XYL, was beneficial for improving the texture of gluten-free oat bread.

**KEYWORDS:** oat, gluten-free, dough, bread, tyrosinase, laccase, xylanase, protein, cross-linking

## INTRODUCTION

Celiac disease is a chronic inflammatory disorder in genetically susceptible subjects, characterized by damage of the small intestinal mucosa caused by wheat gluten and similar alcohol-soluble proteins (prolamines) of barley and rye.[1,2] Oat has recently been approved by the European Commission (EC) as an ingredient in gluten-free labeled products (if cross-contamination from wheat, barley, and rye can be avoided and the gluten content of the oat product remains below 20 mg/kg).[3] Its high content of beneficial fibers ((1→3)(1→4)-$\beta$-D-glucan), proteins, unsaturated fatty acids, vitamins, minerals, and bioactive compounds makes oat flour a healthy alternative for starch-based ingredients in gluten-free breads.

Baking gluten-free bread with adequate textural quality is challenging, because the network formed by gluten proteins is considered to be the most important structure builder in dough. However, the rheological properties of dough and bread could significantly be altered by oxidative enzymes, with subsequent improvement of specific volume and softness of both wheat-based[4,5] and gluten-free breads,[6−8] especially in combination with xylanase (XYL).

Xylanase (endoxylanases EC 3.2.1.8, XYL) cleaves the xylan backbone of arabinoxylan (AX), subsequently increasing the solubility of water-unextractable arabinoxylan (WUAX). The water-unextractable nature of the AX is due to a combination of noncovalent interactions and covalent bonds with neighboring AX molecules, proteins, cellulose, and lignin.[9] Concomitantly, XYL also reduces the molecular weight of the water-extractable arabinoxylan (WEAX).[10] WEAX consists mainly of linear molecules behaving as semiflexible coils in solution.[11] XYL may improve the bread-baking quality by solubilization of WUAX, increased viscosity of dough aqueous phase by WEAX, and possibly the subsequent water redistribution from WUAX to gluten.[10]

The enzymes generating covalent bonds within or between cereal biopolymers are interesting, as the covalent linkages can contribute remarkably to the viscoelastic properties of dough and bread. Depending on the enzymes used, either carbohydrates or proteins or both can be cross-linked, as reviewed by Buchert et al.[12] Tyrosinase (EC 1.14.18.1, TYR) and laccase (benzenediol:oxygen reductase, EC 1.10.3.2, LAC) are oxidative enzymes, which are capable of catalyzing cross-linking biopolymers via their phenolic moieties.

LAC catalyzes oxidation of a variety of organic substrates with concomitant reduction of molecular oxygen to water. LAC can oxidize various aromatic compounds such as substituted mono- and polyphenols and aromatic amines and thiols, producing reactive radicals. Further reactions of radicals may result in cross-linking of monomers, degradation of polymers and ring cleavage of aromatics.[13] LAC is able to cross-link WEAX via ferulic acid (FA) moieties in wheat[14−16] and whole grain oat dough.[7] The cross-linking of WEAX induced by LAC may lead to the formation of WUAX or to the formation of higher molecular weight WEAX, depending on the sites and amount of cross-links formed. The thiols of cysteine and glutathione may reduce the phenoxy radicals, formed by LAC, back to original FA with concomitant oxidation to disulfides.[16,17] Heteroconjugate formation between tyrosine-containing peptides or proteins and feruloylated AX has also been reported by LAC.[18]

TYR can catalyze hydroxylation of monophenols (e.g., *p*-coumaric and caffeic acid, but not FA)[19] to *o*-diphenols and subsequent oxidation of these to *o*-quinones.[20] Thus, TYR can accept both mono- and diphenols as substrates. Quinones may further react nonenzymatically to produce mixed melanins and heterogeneous polymers. Tyrosine side chains in proteins can be oxidized by TYR, and lysyl, tyrosyl, cysteinyl, and histidinyl

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**Table 1. Enzyme Activities of LAC, TYR, and XYL Preparations Used in This Work**

| enzyme | LAC | TYR | protease | XYL | endoglucanase | β-glucanase | α-amylase |
|---|---|---|---|---|---|---|---|
| LAC (nkat/mL) | 7800 | nd[a] | 25 | 0 | 0 | 2.3 | 0 |
| TYR (nkat/mL) | nd | 765 | 0 | 32 | 0 | 6.3 | nd |
| XYL (nkat/g) | nd | nd | 0 | 132600 | 52 | 0 | 0 |

[a] Not determined.

moieties may react further with TYR-oxidized tyrosine residues.[12,21,22] TYR has been shown to be clearly more effective in cross-linking of wheat gliadin than LAC, which was assumed to be the main reason for improved softness and volume of the breads.[4]

The effects of TYR on oat proteins, oat dough, and bread are considered herein for the first time. The aim of this study was to investigate the potential of LAC and TYR, either alone or together with XYL, to improve the structure of gluten-free oat bread. Oat proteins, microstructure, and rheology of doughs were characterized to better understand the effects of these enzymes on dough components and on the structural properties of gluten-free oat bread.

## MATERIALS AND METHODS

**Raw Materials.** The endosperm oat flour from kiln-dried oats was obtained from Helsingin Mylly Ltd., Järvenpää, Finland, and potato flour from Periva Ltd., Finland. Endosperm oat flour contained 8.6 g of moisture, 12.3 g of protein (N = 6.25, db), 1.1 g of ash (db), 5.8 g of dietary fiber (db), and 1.3 g of β-glucan/100 g of flour (db), respectively. The farinograph water absorption was 75.8% (14% moisture). All chemical analyses were made in duplicate. The standard methods[23] used were moisture content, 44-15.02; crude protein, 46-12.01; ash, 08-01.01; total dietary fiber, 32-05.01; β-glucan, 32-23.01; and farinograph, 54-21.01A.

**Enzymes and Enzyme Activity Measurements.** LAC was produced by the white-rot fungus *Trametes hirsuta* and partially purified by anion exchange chromatography.[24] The activity of LAC was measured with 2,2′azinobis(3-ethylbenzthiazoline-6-sulfonic acid ) (ABTS) as substrate.[25] One nanokatal corresponds to the oxidation of 1 nmol of ABTS/s at 25 °C and pH 4.3. TYR was from the filamentous fungus *Trichoderma reesei*, overexpressed, produced, and purified as described by Selinheimo et al.[26] The activity of TYR was measured according to the method of Robb,[27] using 15 mM 3,4-dihydroxy-L-phenylalanine (L-dopa) as substrate. XYL was a commercial product (Pentopan Mono BG, Novozymes A/S, Bagsvaerd, Denmark). The activity of XYL was determined using birch glucuronoxylan as substrate.[28] The activity of β-glucanase was measured with a method described by Zurbriggen et al.[29] Endoglucanase,[30] α-amylase,[31] and protease (Protazyme AK tablets, Megazyme International Ireland Ltd.) activities were also determined from the enzyme preparations. The TYR preparation had a low XYL side activity, and the XYL preparation had a low endoglucanase side activity (Table 1).

**Fractionation of Oat Flour Proteins and Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE) Analysis.** SDS-PAGE analysis of oat protein fractions was performed with and without incubation with LAC or TYR. Oat protein fractions were extracted from endosperm oat flour (Helsingin Mylly Ltd., Finland), which was defatted by extracting the flour with acetone (40 g of flour/L) for 30 min with continuous stirring. Albumin and globulin were isolated with the sequential extraction described by Mikola and Jones,[32] and prolamin was isolated according to the method of Mikola et al.[33] Albumin was freeze-dried; globulin and prolamin were frozen at −20 °C. The protein contents of fractions were analyzed by using the Lowry method (DC protein assay, Bio-Rad, Hercules, CA) with bovine serum albumin as standard. Protein fractions (3 mg/mL) were suspended and treated in 200 mM sodium citrate buffer, pH 6.0; 0.2 M NaCl was added to the buffer, which contained a globulin fraction. LAC or TYR (100 or 500 nkat/g protein) was added to the protein suspensions and incubated at 45 °C for 2 h. The enzyme levels were chosen on the basis of earlier results obtained by Flander et al.[7] and Selinheimo et al.[4] A reference sample without enzyme addition was prepared from each fraction. After incubation, the reaction was stopped by adding 1 volume (1:2) of SDS-PAGE sample buffer with 1% (w/v) DTT (Sigma, Germany) and boiling for 2 min. Prolamin samples were boiled for 5 min. A 12% Tris-HCl gel (Bio-Rad) was used for albumin and globulin and a 16−18% Tris-HCl gradient gel (Bio-Rad) for prolamin. The molecular weight mass standard with a range of 21.5−113 kDa was used (prestained low range, catalog no. 161-0305, Bio-Rad).

**Preparation of Doughs for Microscopical and Rheological Analysis.** The amount of water added to the doughs was determined according to the water absorption of the farinograph at a consistency of 500 BU. The flour content of the endosperm oat dough was 9.46 g, and the water content was 7.17 g. TYR (0, 10, and 30 nkat/g flour) was added to the water just before mixing with flours. The doughs were mixed with a farinograph (Brabender, Duisburg, Germany) at 25 °C for 4 min at slow speed (63 rpm).

**Microscopical Examination of the Doughs.** The dough cubes (each side of the cube ∼0.5 cm) were prepared as described previously.[4] Sections of 4 μm were cut by using a Leica rotary microtome HM 355 (Leica, Heidelberg, Germany) and transferred to glass slides. The sections were stained either with protein-sensitive 0.2% Xylidine Ponceau (Gurr, BDH Chemicals Ltd.) or with 0.1% Acid Fuchsin (Gurr, BDH Chemicals Ltd., Poole, U.K.) and 0.01% Calcofluor (Fluorescent Brightener 28, Aldrich, Germany). Both Xylidine Ponceau and Acid Fuchsin stain proteinaceous structures red, whereas Calcofluor stains cell walls (mainly β-glucan) blue. The stained sections were examined using an Olympus BX-50 microscope (Olympus Corp., Tokyo, Japan) connected to a PCO SensiCam CCD color camera (PCO AG, Kelheim, Germany) with Cell^P imaging software (Olympus), which was also used for image analysis.

To validate the visual observations, the particle size distributions of structures stained with Xylidine Ponceau were analyzed from 20 images/dough by classifying particles according to their area and number. The proteinaceous particles were divided into four different classes indicated by false colors: 2−10 $\mu m^2$ (red), 10−100 $\mu m^2$ (green), 100−1000 $\mu m^2$ (blue), and 1000−5000 $\mu m^2$ (yellow). The effect of TYR dosage on protein aggregation was analyzed by counting the mean area and mean count of protein particles between 100 and 1000 $\mu m^2$/treatment (Figure 3). Similar particle analysis of protein and cell wall structures was performed in the sections stained with Acid Fuchsin and Calcofluor (10 images/dough).

**Rheological Tests of the Dough.** Large strain dough extensibility was measured using the Kieffer dough extensibility test.[34] After mixing in the farinograph, the 16.63 g doughs were molded by hand to fit to the press immediately after mixing. The press was transferred to a resealable polyethylene bag (Minigrip) and was kept in an incubator (Salvis Thermocenter 2401, E. Renggli AG, Rotkreuz, Switzerland) at 30 °C for 40 min to allow stress relaxation before measurements. Six to

**Table 2. Recipe of the Gluten-free Oat Bread**

| ingredient | g | % of flour weight |
|---|---|---|
| endosperm oat flour | 995.0 | 75.8 |
| potato starch | 318.3 | 24.2 |
| water | 1172.0 | 89.2 |
| sugar | 38.8 | 2.9 |
| salt | 24.9 | 1.9 |
| margarine | 38.8 | 2.9 |
| baker's yeast | 41.0 | 3.1 |
| DATEM[a] | 7.6 | 0.6 |
| CMC[b] | 8.8 | 0.7 |
| total | 2645.2 | |

[a] Panodan A2020, Danisco A/S, Grinsted, Denmark. [b] Cekol 50000, Cp Kelco Ltd., Aanekoski, Finland.

eight dough strings/dough were obtained from the press. Rheological properties were measured for 14 replicate dough strings/TYR dosage using a Kieffer dough extensibility rig fitted onto a TA.XT2 Texture Analyzer (Stable Micro Systems Ltd., Godalming, U.K.) and equipped with a 5 kg load cell. The crosshead speed was 3.3 mm/s.

**Baking Procedures.** The effects of enzymes on the specific volume and firmness of the gluten-free oat breads were studied by baking breads with a straight dough baking method in duplicate. The recipe for the breads is presented in Table 2. LAC (14 nkat/g flour) or TYR (20 nkat/g flour) was added to the water immediately prior to mixing with flours, and XYL (20 nkat/g flour) was mixed with flour. All dry ingredients were mixed together and placed in a spiral mixer (Diosna SP 12 F, Dierks and Söhne GmbH, Osnabrück, Germany). The yeast (Finnish Yeast Ltd., Lahti, Finland) was suspended in water (26 °C) and added to the dry ingredients with the tempered shortening (Sunnuntai, Raisio Group plc, Raisio, Finland). The dough was mixed at low speed (100 rpm) for 2 min and at fast speed (200 rpm) for 5 min. After intermediate proofing at room temperature (20 °C) for 20 min, the dough was divided into six 400 g pieces, rounded, molded, and placed in tempered pans that had been sprayed with pan grease and proofed at 80% relative humidity and 37 °C for 45 min. The breads were baked at 220 °C for 40 min and with 15 s of steam at the beginning. After baking, the loaves were cooled for 2 h before being weighed. Specific volumes of the breads were measured with BreadVolScan (Backaldrin, Asten, Austria) from six replicates. Crumb hardness was measured at 2 and 48 h after baking with a TA-XT2 Texture Analyzer (Stable Micro Systems) using the texture profile analysis (TPA) test. Six 25 mm thick slices were used for the analysis; two slices were taken from each of the three different breads. The crust of the slices was removed so that only the textural parameters of the crumbs were measured. The slices were compressed by 10 mm (40%) with a speed of 1.7 mm/s. The results are presented as an average of six replicate loaves baked on two different days.

**Statistical Analysis.** One-way analysis of variance (ANOVA) and the Tukey test were performed to study the significant differences ($p < 0.05$) between means of different enzymatic treatments using the statistical program SPSS 17.0.1 for Windows (SPSS Inc., Chicago, IL).

## RESULTS AND DISCUSSION

**Effects of Tyrosinase and Laccase on Oat Protein Fractions.** The ability of TYR to cross-link isolated oat protein fractions was studied by the SDS-PAGE analysis and compared to the LAC-treated oat proteins. TYR was able to cross-link the globulins of oat effectively, as visualized by the formation of higher molecular weight products in the gel (Figure 1). The

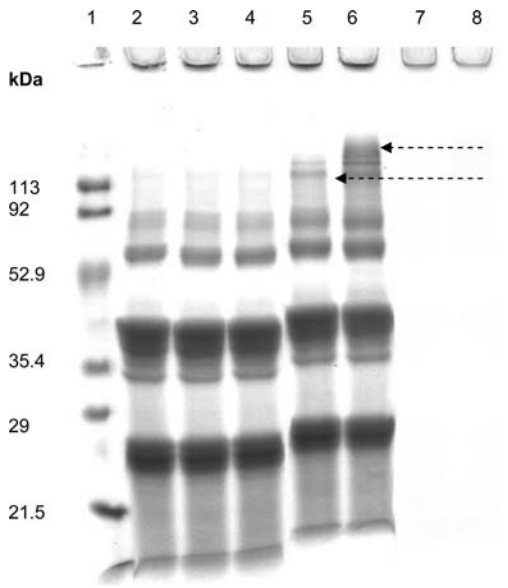


**Figure 1.** Reduced SDS-PAGE profile of LAC- or TYR-treated oat protein fractions. Enzyme dosages (nkat) are per gram of protein. Lanes: (1) molecular weight marker; (2) reference; (3) with 100 nkat LAC; (4) with 500 nkat LAC; (5) with 100 nkat TYR; (6) with 500 nkat TYR; (7) enzyme control, LAC, 500 nkat (without oat protein); (8) enzyme control, TYR, 500 nkat (without oat protein).

efficiency of cross-linking increased as a function of TYR dosage (Figure 1, lanes 5 and 6). LAC did not affect the molecular weights of globulins. Neither TYR nor LAC affected the molecular weights of albumins or prolamins (data not shown). Selinheimo et al.[4] reported similar results with LAC- and TYR-treated wheat proteins. TYR (100 nkat/g protein) was clearly more effective in cross-linking wheat gliadins than LAC (cross-linking was detected only at a dosage of 10000 nkat/g protein).[4]

**Effect of Tyrosinase on the Microstructure of Oat Dough.** The effects of the TYR treatment on different flour components, that is, protein, cell wall (e.g., $\beta$-glucan), and starch, were studied by microscopy with two different staining procedures (Figure 2). The proteins were stained red with 0.2% Xylidine Ponceau in slides 1A−C of Figure 2. With the TYR-treated dough (30 nkat TYR/G flour) (Figure 2, slide 1C), the result was the formation of large red areas. As can be seen from Figure 3, the count and area of protein particles with sizes between 100 and 1000 $\mu m^2$ (false-colored as blue in Figure 2 slides 2A−C) of the dough with 30 nkat TYR/g flour were significantly ($p < 0.05$) higher than the count and area of particles in the control dough. On the basis of the results of SDS-PAGE and microscopical analysis, it is suggested that these blue areas are probably aggregated globulins formed by TYR-induced cross-linking between the oat proteins. TYR also affected the cell walls, which consist mainly of $\beta$-glucan in oat.[35,36] The area and count of particles (with diameter from 10 to 1000 $\mu m^2$) dyed with Calcofluor decreased significantly ($p < 0.05$) as the dosage of TYR increased (Figure 2, slides 3A−C). This may reflect slight degradation of cell walls by the side activities of xylanase and $\beta$-glucanase in the TYR preparation.

**Effects of Tyrosinase on Dough Rheology.** The effects of TYR on the rheological properties of oat dough were studied by a large deformation rheology measurement, the Kieffer test. The measurement hook extends the dough string until its elastic limit is exceeded and it ruptures; thereby, the maximum resistance

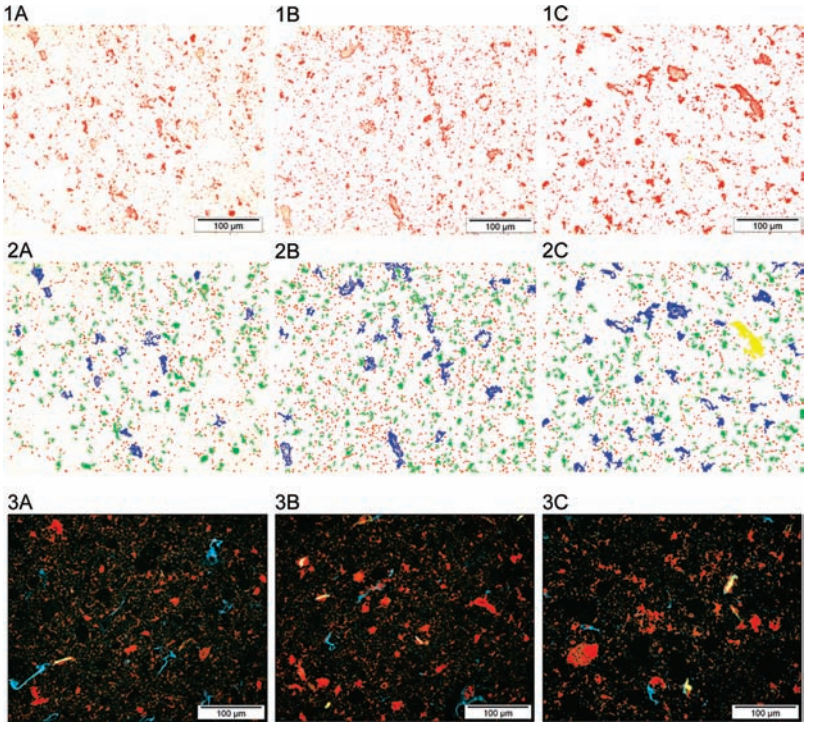


**Figure 2.** Microscopy of oat doughs treated with tyrosinase (scale bar = 100 $\mu$m): (1) stained with Xylidine Ponceau, 0.2%, protein dyed red; (2) particle size distribution of the proteins in images stained with Xylidine Ponceau. (protein particles > 1000 $\mu m^2$ are presented in yellow, those = 100−1000 $\mu m^2$ are presented in blue, those = 10−100 $\mu m^2$ are presented in green, and 4−10 $\mu m^2$ particles are presented in red color); (3) 0.1% Acid Fuchsin and 0.01% Calcofluor, cell walls dyed blue and protein red. Treatments: (A) reference; (B) TYR 10 nkat/g of flour; (C) TYR 30 nkat/g of flour.

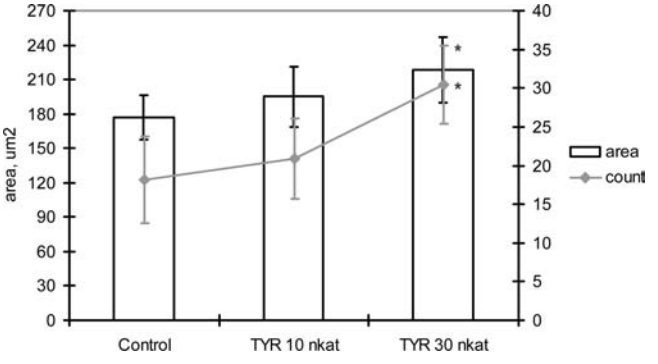


**Figure 3.** Image analysis of protein particles between 100 and 1000 $\mu m^2$ from microscopical images stained with Xylidine Ponceau. Columns represent the mean area of the protein particles/image, and the line represents the mean count of protein particles/image. Mean values ($n = 20$) accompanied by an asterisk are significantly different ($p < 0.05$).

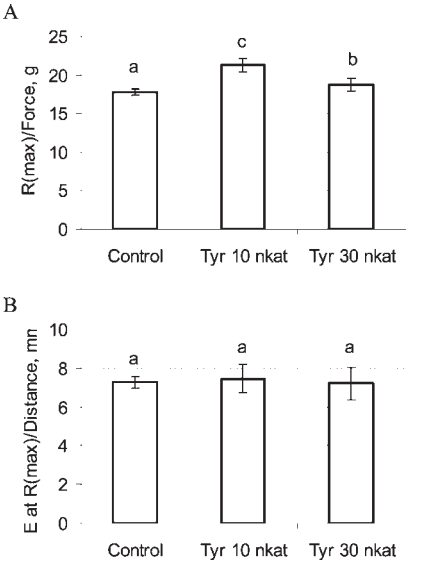


**Figure 4.** Effect of TYR on rheological properties of oat dough: (A) $R$(max) = maximum resistance to the extension; (B) E at $R$(max) = extensibility at $R$(max). All columns represent the mean values of eight replicates. Mean values accompanied by a different letter are significantly different ($p < 0.05$).

(a peak force) and the extensibility (distance of the hook from the start point) of the dough string are recorded. TYR increased the hardness of the oat dough, as indicated by an increase in the resistance to extension (Figure 4A). The SDS-PAGE assay showed that the oat globulins were significantly affected by TYR (Figure 1), which could explain the hardening of the oat dough by TYR. Similar results have been reported in wheat dough with TYR.[4] Microscopical examination confirmed an aggregation of the oat proteins caused by TYR (Figure 3). Addition of 30 nkat TYR/g flour resulted in a softer oat dough than the dough with 10 nkat TYR/g flour (Figure 4A), but harder than the control dough. Such an effect has not been reported with TYR-treated wheat dough[4] or with LAC-treated oat dough.[7,8] Microscopical analysis indicated that degradation of cell walls increased as the dosage of TYR increased (Figure 2, slides 3A−C). At a dosage of 30 nkat/g flour, TYR may have contained enough side activity of XYL and $\beta$-glucanase (1.26 and 0.24 nkat/g flour,

**Table 3. Effects of TYR, XYL, and Their Mixture on Specific Volume and Hardness (after 0 and 2 Days) of the Gluten-free Oat Breads[a]**

| | Control | TYR | XYL | TYR+XYL |
|---|---|---|---|---|
| Photographs of the breads |  |  |  |  |
| Spec.vol., $cm^3/g$ | 2.62 a | 2.80 bc | 2.73 ab | 2.84 c |
| Hardness, 0 day, kg | 0.27 ab | 0.17 a | 0.31 bc | 0.21 a |
| Hardness, 2 days, kg | 0.80 b | 0.73 b | 0.76 b | 0.61 a |

[a] All data represent the mean values of 12 replicates. Mean values followed by a different letter in the same row are significantly different ($p < 0.05$).

**Table 4. Effects of LAC, XYL, and Their Mixture on Specific Volume and Hardness (after 0 and 2 Days) of the Gluten-free Oat Breads[a]**

| | Control | LAC | XYL | LAC+XYL |
|---|---|---|---|---|
| Photographs of the breads |  |  |  |  |
| Spec.vol., $cm^3/g$ | 2.59 a | 2.63 ab | 2.61 ab | 2.69 b |
| Hardness, 0 day, kg | 0.21 a | 0.22 a | 0.23 a | 0.21 a |
| Hardness, 2 days, kg | 0.67 a | 0.76 a | 0.77 a | 0.72 a |

[a] All data represent the mean values of 12 replicates. Mean values followed by a different letter in the same row are significantly different ($p < 0.05$).

respectively) to soften the dough, but this did not overcome the effect of protein cross-linking, because the dough was still significantly harder than the control dough. Renzetti et al.[8] observed increased softening of oat dough with increasing dosage of LAC, which was assumed to be due to the $\beta$-glucanase side activity of the LAC preparation. The TYR did not affect the extensibility of the dough. A similar result was previously reported with a whole grain oat dough and LAC.[7] By contrast, tyrosinase has been reported to decrease the extensibility of wheat dough.[4] Oat dough has very limited extensibility when compared to wheat dough.

**Effects of Tyrosinase, Laccase, and Xylanase on Bread Characteristics.** Effects of TYR, LAC, and XYL, either alone or in combination, on the quality parameters of gluten-free oat bread were studied by measuring the specific volume and instrumental hardness of the breads. TYR, alone and together with XYL, significantly ($p < 0.05$) increased the specific volume of oat breads (Table 3). After 2 days of storage, bread with a combination of TYR and XYL retained its softness significantly better than other breads ($p < 0.05$) (Table 3). This is in accordance with the effects of TYR alone and in combination with XYL on wheat bread,[4] except that fresh wheat breads prepared with a combination of TYR and XYL were also softer than control bread. The cross-linking of oat globulins by tyrosinase may have strengthened the protein network of oat dough, resulting in increased specific volume of oat bread. XYL alone did not affect the specific volume or softness of the gluten-free oat bread. When XYL was added together with TYR, the degradation of AX by XYL, together with polymerization of oat globulins, may have contributed to the softer bread crumb after 2 days of storage.

LAC did not affect the specific volume of oat bread (Table 4). Similar results have been obtained with whole grain oat bread[7] and oat bread with the addition of 0.01% LAC of flour weight (corresponding to a dosage of 1.7 nkat/g flour), although with 0.1% addition LAC increased the specific volume of oat bread.[8] When LAC was combined with XYL, the specific volume of gluten-free oat bread increased significantly (Table 4). This is in accordance with earlier results obtained with whole grain oat bread,[7] in which the combined degradation of $\beta$-glucan by LAC and AX by XYL was assumed to be the main reason for improved volume of the bread.

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## ■ ABBREVIATIONS USED

TYR, tyrosinase; LAC, laccase; XYL, xylanase; AX, arabinoxylan; WEAX, water-extractable arabinoxylan; WUAX, water-unextractable arabinoxylan.

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