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The Bread Dough Stability Improving Effect of Pyranose Oxidase from *Trametes multicolor* and Glucose Oxidase from *Aspergillus niger*: Unraveling the Molecular Mechanism

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ABSTRACT: Glucose oxidase (GO) and pyranose oxidase (P_2O) improve dough stability and bread quality. We here studied whether their mode of action resides in cross-linking of proteins and/or arabinoxylan (AX) molecules through the production of H_2O_2. Evidence for both was deduced from a decrease in extractability of protein and AX from dough made with P_2O, GO, or H_2O_2, using sodium dodecyl sulfate containing buffer and water, respectively. The addition of H_2O_2, P_2O, or GO to a glutathione solution sharply decreased its sulfhydryl (SH) content. P_2O or GO can trigger protein cross-linking through the formation of disulfide (SS) bonds. As a result thereof, SH/SS interchange reactions between low molecular mass SH containing compounds and gluten proteins can be hampered. Furthermore, a decrease in the level of monomeric ferulic acid (FA) esterified to AX in dough points to a role of FA bridges in cross-linking of AX molecules. Our results indicate that the molecular mechanism of dough and bread improvement by P_2O and GO resides in cross-linking of gluten proteins and AX by formation of H_2O_2. They furthermore show that the extent of cross-linking upon addition of P_2O or GO strongly depends on the concentration (and production rate) of H_2O_2.

KEYWORDS: *oxidizing enzymes, gluten proteins, arabinoxylan, glutathione, ferulic acid, tyrosine*

INTRODUCTION

The quantity as well as the intrinsic quality of gluten proteins affect the dough and breadmaking quality of wheat flour.[1,2] Disulfide (SS) bridges in and between gluten proteins are crucial to gluten networks and the viscoelastic properties of dough.[3,4] An optimal level of sulfhydryl (SH) oxidation is essential. Underoxidized dough insufficiently holds the CO_2 produced by the yeast, while its overoxidation hampers gas cell expansion and, hence, reduces bread loaf volume.[4] While Tilley and co-workers postulated cross-linking of tyrosine (TYR) moieties to be important for the development and quality of the gluten network structure,[5] later literature doubts the importance of TYR cross-links in dough.[6,7]

Next to gluten, arabinoxylan (AX) plays a dual functional role in dough and bread quality. First, water extractable (WE-) AX molecules increase the viscosity of the aqueous dough phase, thereby increasing the stability of the liquid films surrounding the gas cells. Second, cross-linking of AX may reinforce the primary gluten network.[8−10] Labat and co-workers reported no covalent bond formation between AX and proteins in model systems and therefore suggested that gluten proteins and AX form two distinct networks.[11] However, some authors described formation of cross-links between TYR residues on gluten proteins and ferulic acid (FA) residues of AX molecules in dough systems, although they only occur at very low levels.[12,13]

It is common practice to add chemical agents or enzymes to bread recipes in order to improve dough and bread characteristics. One class of such improvers is oxidizing agents or enzymes. Oxidizing enzymes such as glucose oxidase (GO, E.C. 1.1.3.4) can improve dough handling properties and dough stability during breadmaking.[14,15] Dunnewind and co-workers found a more extensive cross-linking of gluten proteins in dough made with GO inclusion in the recipe.[16] Likewise, Steffolani and co-workers described the formation of large protein aggregates when adding GO to a dough recipe.[17] In addition, Primo-Martin and co-workers hypothesized that GO also cross-links AX molecules.[18]

Pyranose oxidase (P_2O, E.C. 1.1.3.10) and GO show a similar reaction mechanism.[19] However, while the effect of GO in dough systems has already been extensively studied by several authors, only little data are available on the effect of P_2O. In this study, we report on the effect of P_2O from *Trametes multicolor* on the formation of gluten and/or AX aggregates in dough. As previously shown, P_2O from *T. multicolor* and GO from *Aspergillus niger* have different biochemical characteristics.[19] P_2O from *T. multicolor* shows a higher affinity toward its substrates than GO from *A. niger* does, and different reaction products are formed [2-keto-D-glucose or D-glucono-1,5-lactone for the reaction catalyzed by P_2O or GO, respectively].[19]

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P_2O may be a promising alternative for GO. We here compared the molecular effect of P_2O from *T. multicolor* to that of GO from *A. niger* in *in vitro* and dough systems. Unraveling the complexity of the molecular phenomena induced by these enzymes can serve as a sound basis for application of P_2O and a more directed use of GO by the breadmaking/food industry.

MATERIALS AND METHODS

Materials. GO from *A. niger* was purchased from Sigma-Aldrich (Bornem, Belgium). P_2O from *T. multicolor* was recombinantly produced in *Escherichia coli* and purified as in Decamps and co-workers.[19] The activity of horseradish peroxidase (POD) was expressed in purpurogallin units (PU). In the presence of H_2O_2, POD oxidizes pyrogallol to purpurogallin and H_2O. One PU is defined as the POD level which produces 14 μmol purpurogallin in 1 min at 20 °C. Commercial Kolibri flour from Meneba (Rotterdam, The Netherlands) was used in dough experiments. All chemicals, solvents, and reagents used in the experiments were of at least analytical grade and from Sigma-Aldrich unless specified otherwise.

Standard Analyses. Flour moisture and protein content were measured in triplicate according to AACC International Approved Method 44-15.02[20] and a Dumas combustion method, adapted from AOAC official method 990.03[21] to an automated Dumas protein analysis system (EAS, VarioMax N/CN, Elt, Gouda, The Netherlands), respectively. Kolibri flour had moisture and protein contents (N × 5.7) of 13.1% and 11.5% [the latter on dry matter base (db)], respectively.

Optimal mixing time and water absorption for Kolibri flour were determined by Mixograph (National Manufacturing, Lincoln, NE) and Farinograph (Brabender, Duisburg, Germany) analyses, respectively, based on the AACC International Approved Methods 54-40.02 and 54-21.01.[20]

P_2O and GO Activity Assay. The activities of P_2O and GO were measured with the 2,2′-azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid) assay.[19] One nanokatal (nkat) of P_2O or GO is the amount of enzyme needed to consume 1 nmol of O_2 per second under the conditions of the assay.

Preparation of Wheat Extracts. A wheat extract of low dry matter content (WE1) was prepared by suspending 1.00 g of wheat flour in 10.0 mL of 50 mM sodium phosphate buffer (pH 6.5). The suspension was shaken for 30 min at 7 °C and centrifuged (15 min, 10,000*g*, 7 °C). The supernatant was used immediately as a source of POD as described in the section Oxidation of Glutathione in the Presence of Wheat Peroxidase and a Nonlimiting Level of Glucose.

A wheat extract of intermediate dry matter content (WE2) was used in *in vitro* experiments. This was prepared by suspending 8.00 g of wheat flour in 20.0 mL of H_2O. The suspension was vortexed for 10 s at room temperature and subsequently transferred to an ice bath, homogenized with an Ultra Turrax for 15 s at 24 000 rpm, followed by 10 s at 3400 rpm and 15 s at 24 000 rpm, and then centrifuged (20 min, 28 000*g*, 4 °C). Here, the supernatant was a source of both glucose and POD activity as described in the section Oxidation of Glutathione, Ferulic Acid, or Tyrosine in the Presence of Wheat Peroxidase and a Limited Level of Glucose.

For viscosity experiments, concentrated wheat extract (WE3) was prepared by suspending 10.0 g of wheat flour in 20.0 mL of 50 mM potassium phosphate buffer (pH 6.5). The suspension was shaken [60 min, 150 strokes per minute (spm), 6 °C] and centrifuged (20 min, 1000*g*, 15 °C). The supernatant was used immediately.

***In Vitro* Oxidation of Glutathione, Tyrosine, and Ferulic Acid.** *Oxidation of Glutathione in the Presence of Wheat Peroxidase and a Nonlimiting Level of Glucose.* The effect of different redox agents on the SH content of a glutathione (GSH) solution was investigated in the presence of wheat POD and added glucose (20 mM). We added glucose in these experiments in excess, so that not glucose but O_2 or GSH would be limiting. Stock solutions of H_2O_2 (94 mM) and KIO_3 (1.40 mM) were produced by dissolving the target compounds in 50 mM sodium phosphate buffer (pH 6.5). Stock solutions of P_2O (150 nkat/mL) and GO (150 nkat/mL) were prepared in 50 mM potassium phosphate buffer (pH 6.5). Prior to adding 100 μL of fresh WE1, GSH, and glucose were dissolved in the 50 mM sodium phosphate buffer (pH 6.5). Appropriate volumes of the stock solutions were added to obtain the desired final concentration of the oxidant or enzyme (i.e., 0.041, 0.19, or 9.4 mM for H_2O_2; 0.14 mM for KIO_3; 15 nkat/mL for P_2O and GO). The concentrations of GSH and glucose in the reaction medium were 0.80 mM and 20 mM, respectively. The final volume of the reaction medium was 1.0 mL. For each oxidant or enzyme, three different samples were prepared starting from the same stock solution. Samples (1.00 mL) were shaken (150 spm, room temperature) for 30 min prior to adding 100 μL of a 0.1% solution of 5,5′-dithio-bis(2-nitrobenzoic acid) (Ellman's reagent[22]) in the sodium phosphate buffer to the samples. The mixtures were vortexed vigorously and then shaken (10 min, 150 spm, room temperature) in the dark. Exactly 45 min after adding the above 5,5′-dithio-bis(2-nitrobenzoic acid) solution, the extinction at 412 nm was measured. A calibration curve was made using 0.00, 0.16, 0.33, 0.49, 0.65, 0.81, and 0.98 mM GSH in the sodium phosphate buffer.

Oxidation of Glutathione, Ferulic Acid, or Tyrosine in the Presence of Wheat Peroxidase and a Limited Level of Glucose. The effect of P_2O or GO on GSH, FA, or TYR was investigated in the presence of wheat POD and a limited level of glucose. Oxidation experiments were performed by mixing 0.75 mL of WE2 and 0.95 mL of aqueous solutions of GSH (1.80 mM), FA (0.80 mM), or TYR (1.43 mM) to a final volume of 1.70 mL. P_2O or GO were added to obtain a final concentration of 4.4 nkat/mL. Due to the differences in Michaelis−Menten constants between P_2O and GO toward glucose and O_2,[19] an 8-fold higher level of GO was needed (i.e., 35.3 nkat/mL measured at nonlimiting glucose concentration) to reach rates of O_2 consumption similar to those obtained with 4.4 nkat P_2O/mL with a low glucose concentration (~0.59 mM, WE2).

The O_2 concentration was monitored using a Burnstein-type O_2 microprobe from Heito (0−5 V, OSD23, Paris, France) connected to Datastudio software (Pasco, Roseville, CA). When working with 4.4 nkat P_2O/mL or 35.3 nkat GO/mL, samples (1.50 mL) were withdrawn when O_2 was no longer detectable. That time point was also chosen to withdraw samples (1.50 mL) from the reaction in which 4.4 nkat GO/mL was added even if O_2 was still detectable in that case. The enzymic reactions were stopped by adding 140 μL of 1.0 M hydrogen chloride. The solutions were then vortexed vigorously and analyzed by reversed phase-HPLC using a YMC ODS AQ column (150 × 4.6 mm, 3 μm particle diameter, AIT France, Houilles, France) equipped with a precolumn of the same material (150 × 4.6 mm, 5 μm particle diameter, AIT France). The separation was conducted with a Dionex Ultimate 3000 system equipped with a diode array detector, autosampler, and Chromeleon 6.8 Chromatography Data System software (Thermo Fischer Scientific, Illkirch Cedex, France). The injection volume was 20 μL. Samples were eluted with 0.1% formic acid/acetonitrile (ACN) [70/30 (v/v)]. The flow rate was 0.70 mL/min. FA was detected by measuring extinction at 320 nm. For determining oxidized GSH (GSSG) and TYR levels, samples were eluted with a 0.1% formic acid/ACN mixture [98/2 (v/v)], and the flow rate was lowered to 0.50 mL/min. GSSG was detected by measuring the extinction at 250 nm and the GSH levels were calculated from the GSSG concentrations.[23] TYR was detected by measuring the extinction at 280 nm.

Doughmaking. Yeastless dough samples were prepared by mixing flour (10.0 or 100.0 g, 14.0% moisture basis) with water to optimum dough consistency at 25 °C in a 10 or 100 g pin mixer (National Manufacturing, Lincoln, NE), respectively. If not used in dough spread analysis experiments, freshly mixed dough samples were frozen, lyophilized, ground using an A10 basic (Ika-Werke, Staufen, Germany) cooled analytical grinder, and sieved (250 μm) to obtain samples for further analysis.

Dough Spread Analysis. Dough spread behavior was studied as in Hoseney and co-workers.[24] Yeastless dough was prepared from 100.0 g of flour as in the above section. Where used, H_2O_2, 2-keto-D-glucose, or D-glucono-1,5-lactone (5.0 μmol/g flour) were added to the dough formula together with the water used for doughmaking.

Immediately after mixing, dough was mechanically rounded, and its height and width (in two directions) were measured. The further part of the procedure was as in Decamps and co-workers, and Δ(width/height) values were calculated.[14]

All spread tests were performed in duplicate on two independently produced dough samples.

Preparation of Dough Extract. Water extraction of ground dough samples was as in Courtin and co-workers.[25] The sample (5.0 g) was accurately weighed in a 250 mL centrifuge tube. Deionized water (100 mL, 6 °C) was added, and the suspensions were shaken (30 min, 150 spm, 6 °C). After centrifugation (15 min, 10 000*g*, 6 °C), the supernatant was transferred to a flask and immediately frozen using liquid N_2. The pellet was suspended in water (50 mL, 6 °C) and centrifuged as described above. The second supernatant was frozen in the same flask. After freeze-drying, boiling water (150 mL) was added to the flask, which was then held in a bath with boiling water for 30 min to inactivate enzymes. The suspension was frozen as described above and freeze-dried. The lyophilized material was dispersed in water (50 mL) and centrifuged (15 min, 10 000*g*, 6 °C) to remove heat-coagulated proteins. The supernatant was frozen until further analysis and is referred to as dough extract.

Viscosity Measurements. Stock solutions of P_2O or GO (both 425 nkat/mL) were prepared in 50 mM potassium phosphate buffer (pH 6.5). To 4.8 mL WE3 (in triplicate, starting from the same stock solution) was added 200 μL of the stock solutions to obtain the desired final enzyme concentration (i.e., 17 nkat/mL for P_2O or GO). The resulting 5.0 mL sample was incubated for 5 min at room temperature.

Viscosities of samples and of dough extracts prepared as described above were measured using an Ostwald type capillary viscosimeter (AVS 400, Schott Geräte, Hofheim, Germany). The relative viscosity is the ratio of the flow time at 30 °C of the sample to that of deionized water.

Determination of Protein Extractability. To determine the level of protein extractable in sodium dodecyl sulfate (SDS) containing a medium, samples containing about 1.0 mg of protein were extracted with 1.0 mL of 50 mM sodium phosphate buffer (pH 6.8) containing 2.0% (w/v) SDS (Acros Organics, Geel, Belgium). To determine the maximum level of extractable proteins in wheat flour, an aliquot containing 1.0 mg of protein was extracted under a N_2 atmosphere with 1.0 mL of the above sodium phosphate buffer (pH 6.8) containing SDS [2.0% (w/v)], dithiothreitol [1.0% (w/v), Acros Organics], and urea (2.0 M). Triplicate samples were shaken for 60 min (150 spm, room temperature) and centrifuged (10 min, 11 000*g*, room temperature). The supernatants were then filtered over a polyethersulfone membrane (0.45 μm, Millex-HP, Millipore, Carrigtwohill, Ireland), and extracted proteins were separated using size exclusion-HPLC. Size exclusion-HPLC was performed as in Lagrain and co-workers using a LC-2010HT system (Shimadzu, Kyoto, Japan) with automatic injection.[26] The extracts (60 μL) were loaded on a BioSep-SEC-S4000 column (300 × 7.8 mm, Phenomenex, Torrance, CA) and eluted with a mixture of ACN and 0.05% trifluoroacetic acid (TFA), prepared by adding the latter to 1.00 L of ACN to obtain a final volume of 2.00 L. The flow rate was 1.0 mL/min. Separation was at 30 °C, and protein elution was monitored at 214 nm.[27] The elution profiles of nonreduced samples were divided into two fractions using the lowest extinction value between the two peaks as a cutoff point. The fractions containing higher and lower molecular mass proteins are further referred to as glutenin and gliadin extractable in SDS containing buffer, respectively. The levels of extractable glutenin and gliadin were calculated from the corresponding peak areas. Simultaneously, the total extractable gluten level in wheat flour was determined by extracting the proteins under reducing conditions as described above. The extractable glutenin and gliadin levels in dough samples were expressed as percentages of the total extractable protein of flour.

Total Ferulic Acid Determination in Wheat Flour and Dough. To triplicate samples (100 mg) of wheat flour or lyophilized dough was added 4.0 mL of 2.0 M sodium hydroxide, and hydrolysis was performed by stirring the suspension for 2 h at 35 °C under a N_2 atmosphere in the dark. Hereafter, samples were acidified with 4.0 M hydrogen chloride to pH 2.00 ± 0.05. An aliquot (25.0 μL) of caffeic acid in methanol (25 μg/mL) was added as an internal standard. After vigorous shaking with 2.0 mL of diethyl ether, the samples were centrifuged for 5 min (200*g*, 20 °C), and the diethyl ether phase was recovered. The diethyl ether extraction was performed twice. The combined ether layers were dried under a N_2 flow, and the residue was dissolved in 250 μL of methanol, filtered over a polyethersulfone membrane (0.45 μm), and injected (20 μL) on a reversed phase-HPLC C18 column (Luna Phenyl-Hexyl, Phenomenex) coupled to a LC-10 AD device (Shimadzu) as in Dobberstein and Bunzel.[28] Separation was at 45 °C and the elution monitored at 280 nm. We used three eluents for the gradient mobile phase: 1.0 mM aqueous TFA, 900 mL of ACN to which 1.0 mM TFA was added to a final volume of 1.00 L, and 900 mL of methanol to which 1.0 mM TFA was added to a final volume of 1.00 L.[28]

Characterization of the Water Extractable Arabinoxylan Population. AX contents were determined in triplicate by gas chromatography as in Courtin and co-workers.[29] Aqueous extracts were prepared by suspending samples (2.00 g) in water (20.0 mL). Following shaking for 30 min (150 spm) at room temperature and centrifugation (10 min, 1000*g*, 7 °C), the supernatants were filtered through paper (MN 615, Machery-Nagel, Düren, Germany) prior to further analysis.

Hydrolysis of AX in 2.5 mL of supernatant, prepared as described above or in 2.5 mL dough extract, was performed at 110 °C for 60 min following the addition of 2.5 mL of 4.0 M TFA. The released monosaccharides were reduced with sodium borohydride and peracetylated with acetic acid anhydride according to Englyst and Cummings.[30] Samples (1.0 μL) were separated on a Supelco SP-2380 column (30 × 0.32 mm, 0.2 μm film thickness, Supelco, Bellefonte, PA) with helium as a carrier gas in an Agilent 6890 series (Wilmington, DE) gas chromatograph equipped with an autosampler, splitter injection port (split ratio 1:20), and a flame ionization detector. Separation was at 225 °C with injection and detection at 270 °C. β-D-Allose was used as an internal standard, and calibration samples containing defined concentrations of L-arabinose, D-xylose, D-mannose, D-galactose, and D-glucose were analyzed and used to calculate their concentrations. Total AX and WE-AX contents (db) were calculated as 0.88 times the sum of xylose and arabinose contents, with correction of the arabinose content for the presence of arabinogalactan peptides.[31]

Statistical Analysis. Spread values, viscosity values, SH concentrations, protein extractability levels, and FA and WE-AX values were analyzed using ANOVA with comparison of mean values using the Tukey test ($P < 0.05$). We used the Statistical Analysis System software (v.8.1, SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

Dough Spread Analysis. Spread values of yeastless dough samples to which the reaction products of P_2O and GO were added were analyzed. H_2O_2 supplementation caused a strong reduction of the spread value [Δ(width/height) of 0.42] relative to that of the control sample [Δ(width/height) of 1.08]. The addition of neither 2-keto-D-glucose [Δ(width/height) of 1.12] nor D-glucono-1,5-lactone [Δ(width/height) of 1.17] had a significant effect on dough spread.

Hence, the effect of P_2O and GO on dough rheology can be ascribed to H_2O_2 and not to the other reaction products of the enzymic reactions catalyzed by P_2O or GO, i.e., 2-keto-D-glucose or D-glucono-1,5-lactone, respectively. The affinity of wheat catalase toward H_2O_2 [K_M of 300−400 mM[32]] is lower than that of wheat POD [K_M of 0.7−1.2 mM[33]]. Furthermore, as H_2O_2 had a profound impact on dough rheology, we hypothesize that the activity of catalase, endogenously present in wheat flour, has only a minor influence.

***In Vitro* Oxidation of Glutathione in the Presence of a Nonlimiting Level of Glucose.** In the presence of a nonlimiting level of glucose and native wheat POD, H_2O_2 (either added or produced by P_2O or GO) oxidized GSH (Figure 1). The higher the level of H_2O_2 (added or produced), the more GSH was oxidized. The addition of 0.19 mM H_2O_2 oxidized about 0.40 mM GSH (Figure 1), in accordance with the stoichiometry, whereby 1 mol of H_2O_2 oxidizes 2 mol of GSH. The addition of 15 nkat/mL P_2O or GO or 9.4 mM H_2O_2 resulted in complete oxidation of the GSH present (Figure 1).

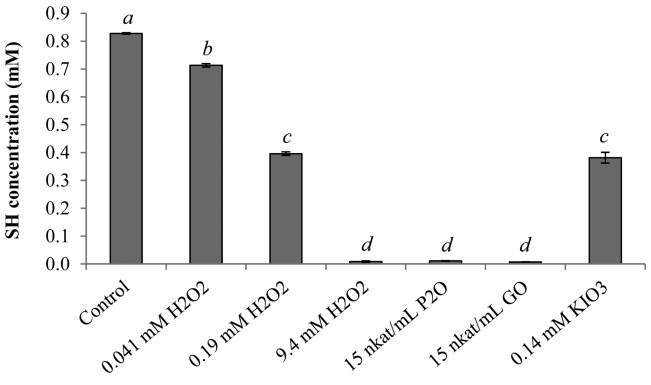

Figure 1. Effect of H_2O_2, pyranose oxidase (P_2O), glucose oxidase (GO), or KIO_3 on the sulfhydryl (SH) content of a reduced glutathione (GSH) solution in the presence of aqueous wheat extract WE1 (see text) and a nonlimiting concentration of glucose (20 mM). Values with the same letter are not significantly different ($P < 0.05$).

KIO_3 was also tested for its GSH oxidizing potential (Figure 1). The addition of 0.14 mM KIO_3 oxidized about half of the initial GSH concentration (i.e., 0.45 mM). This overall ratio (i.e., oxidation of 3 mol of GSH per mol KIO_3) has also been observed by Louarme in the oxidation of cysteine by KIO_3 at pH 5.6.[34] The level of oxidation is lower than expected based on the reaction stoichiometry (i.e., oxidation of 6 mol of GSH per mol of KIO_3).[35] This may be due to the formation of higher oxidation states from cysteine, such as sulfinic and sulfonic acids. This was already previously observed when KIO_3 was used to oxidize cysteine at neutral pH values.[35]

***In Vitro* Oxidation of Glutathione, Tyrosine, and Ferulic Acid in the Presence of a Limited Level of Glucose.** When WE2 was used as a source of both glucose and POD activity, the effect of similar levels of P_2O and GO (i.e., 4.4 nkat/mL, measured at nonlimiting glucose conditions) on oxidation of GSH was more pronounced for P_2O than for GO (Figure 2). The latter can be explained by the large difference in kinetic constants toward glucose between the two enzymes.[19] To obtain a similar oxidation effect on GSH, the GO level had to be increased 8-fold (i.e., 35.3 nkat/mL measured at a nonlimiting glucose concentration). Likewise, a GO level of 4.4 nkat/mL resulted in substantially lower oxidation of FA than did 4.4 nkat P_2O/mL (Figure 2). Only when 35.3 nkat GO/mL was added was the level of FA oxidation equivalent to that obtained when adding 4.4 nkat P_2O/mL to the sample. When GSH and FA were simultaneously added to the reaction medium, they were both oxidized by H_2O_2 produced by P_2O or GO (results not shown). No evidence for preferential oxidation of one of the compounds was found. Finally, under the present conditions, no oxidation of TYR by P_2O or GO was detected (Figure 2).

An earlier study indicated that an optimal P_2O or GO activity level in dough results in increased dough stability and stress tolerance.[14] We speculated that this stability improvement was caused by the effect of P_2O and GO on both gluten and AX. The impact of P_2O and GO and, hence, the nature and level of the oxidized products in a complex dough system can, however, not be directly deduced from *in vitro* approaches. The reaction conditions in dough are very different from those in model systems. For instance, the O_2 concentration in dough is limited, and the mobility of the reactants deviates from that in the above-described model systems. Indeed, while *in vitro* oxidation of GSH and FA upon the addition of P_2O or GO allows speculating on enzyme induced cross-linking of gluten and AX in dough through SS and di-FA bridge formation, respectively, experimental proof in dough systems is needed to confirm this hypothesis.

Extractability of Gluten Proteins in Dough Systems. In the present study, a concentration of P_2O or GO beneficial to dough stability, i.e., 0.51 nkat/g of flour, was used.[14] In addition, a higher level of these enzymes, i.e., 8.7 nkat/g of flour, was used to make their effect more explicit. Furthermore, the level of H_2O_2 (5.0 μmol/g flour) corresponds to that which can be theoretically produced by the oxidation reaction of P_2O or GO assuming complete oxidation of the glucose initially present in flour (about 0.10% of dry weight). However, as O_2 may well be the first limiting substrate, this addition level of H_2O_2 is assumed to be an overdose.

During dough mixing, gluten proteins are stretched and react with one another to form SS bonds.[36] As a result, an extended network is formed. In freshly mixed yeastless dough, no significant changes in glutenin extractability in SDS containing buffer were noted with either 0.51 nkat P_2O or GO/g of flour (Table 1). The addition of P_2O, GO, or H_2O_2 did not impact

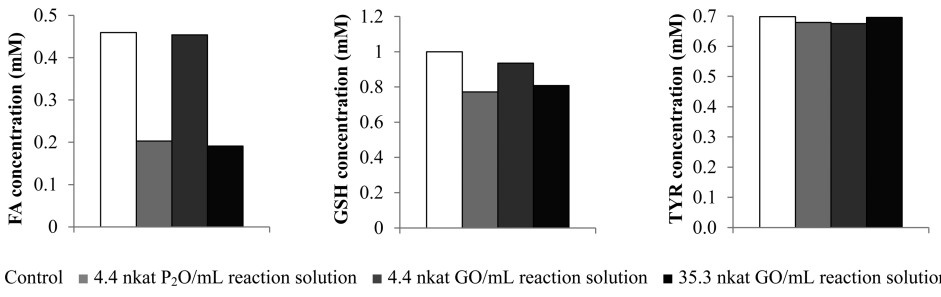

Figure 2. Ferulic acid (FA, 0.45 mM), glutathione (GSH, 1.00 mM), and tyrosine (TYR, 0.75 mM) oxidation in the presence of aqueous wheat extract WE2 (see text) and a limited level of glucose (0.59 mM) upon the addition of pyranose oxidase (P_2O) or glucose oxidase (GO). A higher level of GO that resulted in rates of O_2 consumption similar to those obtained with the added P_2O level was also added.

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Table 1. Extractability in Sodium Dodecyl Sulfate Containing Medium of Proteins in Freshly Mixed Yeastless Control Dough or Dough Containing Added Pyranose Oxidase (P_2O), Glucose Oxidase (GO), H_2O_2, or KIO_3[a]

	sample	glutenin extractability (%)	gliadin extractability (%)
freshly mixed yeastless dough	control (C)	32.8 ± 0.8 *cd*	51.6 ± 0.7 *a*
	C + 0.51 nkat P_2O/g flour	33.6 ± 1.2 *c*	50.7 ± 0.7 *a*
	C + 8.7 nkat P_2O/g flour	28.9 ± 0.5 *e*	49.8 ± 0.8 *a*
	C + 0.51 nkat GO/g flour	33.1 ± 1.7 *cd*	51.2 ± 1.8 *a*
	C + 8.7 nkat GO/g flour	36.9 ± 3.3 *b*	51.8 ± 0.7 *a*
	C + 5.0 μmol H_2O_2/g flour	30.5 ± 0.7 *de*	51.3 ± 0.7 *a*
	C + 20 ppm KIO_3	40.2 ± 0.7 *a*	51.3 ± 1.0 *a*

[a]Values are expressed as % of the total extractable protein of wheat flour. Values with the same letter are not significantly different ($P < 0.05$) per column.

the extractability of gliadin in SDS containing buffer (Table 1). We hypothesize that the addition of low enzyme levels to wheat flour used for doughmaking induces moderate oxidation of protein SH groups. The remaining free SH groups on proteins and low molecular weight SH containing compounds can then still take part in interchange reactions between SH groups and SS bonds. The thus formed extended gluten network is reinforced by the additional SS cross-links formed and logically can better withstand stress during processing than the network in control dough.

Glutenin extractability of yeastless dough tended to be lower when H_2O_2 (5.0 μmol/g flour) was added to the dough recipe (Table 1). Furthermore, the addition of a high level of P_2O (8.7 nkat/g flour) significantly reduced glutenin extractability (Table 1). In contrast, glutenin extractability of freshly mixed yeastless dough made with the addition of 8.7 nkat GO/g of flour or with the addition of 20 ppm KIO_3 was significantly higher than that of control dough (Table 1). We hypothesize that, under strongly oxidizing conditions, the majority of the SH groups are quickly oxidized. Only minor levels of free SH groups remain, and interchange reactions between SH groups and SS bonds are hampered by the low level of free SH groups. It would then seem logical that, during mixing, proteins will not very easily spread out, and that smaller gluten aggregates rather than an extended gluten network are formed. Such aggregates would then be far less effective in stabilizing the dough structure than those formed upon the addition of low enzyme levels as their smaller size thwarts them to support the entire dough matrix. The here observed significant differences between the effect of high levels of P_2O and GO on glutenin extractability in SDS containing buffer can probably be explained by the differences in kinetic constants between P_2O and GO, which result in higher production levels of H_2O_2 when using P_2O.[19] Indeed, after creating small gluten aggregates, as described above, P_2O may well cross-link these small aggregates into larger, unextractable glutenin aggregates.

Veraverbeke and co-workers found an increased glutenin extractability in SDS containing buffer in mixed dough when supplementing flour with 26 ppm KIO_3 (on flour basis).[37] These authors attributed such an increase to higher dough stiffness and, as a result, a more extensive breakage of (non-) covalent bonds upon shear forces. Other possibilities would be stress induced scission of SS bonds of higher molecular mass glutenin polymers or oxidation of small SH containing compounds, eliminating their potential involvement in exchange reactions between SH groups and SS bonds. Taken together, "smaller" gluten aggregates would be formed which would still be extractable in SDS containing buffer. There are different theories on (the extent of) gluten cross-linking in dough containing added GO. Rasiah and co-workers reported almost no additional cross-linking of glutenin molecules at relatively low GO levels.[38] However, Bonet and co-workers observed cross-linking of high molecular mass glutenin subunits with low to moderate GO levels.[15] Steffolani and co-workers described the formation of large protein aggregates when adding high GO levels to flour used for doughmaking.[17] Whatever the case, protein cross-linking heavily depends on flour properties and enzyme addition levels.

Taken together, we hypothesize that the extent of gluten cross-linking depends on the concentration of H_2O_2 formed or added. In addition, for P_2O and GO, the production rate of H_2O_2 would also play an important role in determining the final gluten conformation.

***In Vitro* Viscosity Experiments.** The relative viscosity of WE3 increased significantly when supplementing it with P_2O or GO (17 nkat/mL; Table 2). To determine the importance of wheat endogenous POD, WE3 was also boiled for 30 min before being used in parallel viscosity experiments (Table 2). As expected, boiling of WE3 nullified the effect of P_2O on the relative viscosity, while the addition of horseradish POD (8.7 PU/mL) to boiled WE3 (partially) restored the effect of P_2O on the relative viscosity.

Table 2. Relative Viscosity of Wheat Extract (WE3) and WE3 to Which Pyranose Oxidase (P_2O), Glucose Oxidase (GO), or Horseradish Peroxidase (POD) Was Added[a]

sample	relative viscosity
WE3	4.99 ± 0.24 *b*
WE3 + 17 nkat P_2O/mL	6.71 ± 0.84 *a*
WE3 + 17 nkat GO/mL	6.12 ± 0.52 *a*
boiled WE3	4.57 ± 0.02 *B*
boiled WE3 + 17 nkat P_2O/mL	4.28 ± 0.02 *B*
boiled WE3 + 8.7 PU POD/mL	4.25 ± 0.08 *B*
boiled WE3 + 8.7 PU POD/mL + 17 nkat P_2O/mL	5.02 ± 0.14 *A*

[a]Viscosity is expressed as relative viscosity towards water as a blank. Values with the same letter are not significantly different ($P < 0.05$).

The observed viscosity increase suggests cross-linking of WE-AX molecules in the presence of H_2O_2, which itself is formed in the enzymic reaction catalyzed by P_2O or GO.

Cross-Linking of Arabinoxylan Molecules in Dough Systems. The total FA level in wheat flour (i.e., both free and AX-bound FA not involved in di- or tri-FA bonds) was 95 ppm (i.e., about 420 nmol/g of flour db). There were no significant differences in total FA or WE-AX levels of freshly mixed yeastless dough made with the addition of 0.51 nkat P_2O or GO/g flour (Table 3). Probably, the theoretical level of H_2O_2 produced by P_2O or GO during mixing (i.e., 78.5 nmol/g flour, no O_2 or glucose limitation, 30 °C, and pH 6.5) was too low to exert a pronounced effect on the FA concentration as it would seem logical that only part of the H_2O_2 produced would be used to oxidize FA. However, yeastless dough made with 8.7 nkat P_2O or GO/g of flour significantly decreased both the

Table 3. Total Ferulic Acid (FA, % on db) and Water Extractable Arabinoxylan (WE-AX, weight % on db) Levels in Control Wheat Flour, Control Freshly Mixed Yeastless Dough, or Freshly Mixed Dough Containing Added Pyranose Oxidase (P_2O), Glucose Oxidase (GO), or H_2O_2[a]

	sample	FA (ppm on db)	WE-AX (weight % on db)
wheat flour		95.0 ± 5.7	0.42 ± 0.03
freshly mixed yeastless dough	control (C)	82.0 ± 4.7 *a*	0.57 ± 0.05 *a*
	C + 0.51 nkat P_2O/g flour	81.3 ± 3.5 *a*	0.57 ± 0.11 *a*
	C + 8.7 nkat P_2O/g flour	55.5 ± 2.1 *b*	0.30 ± 0.01 *b*
	C + 0.51 nkat GO/g flour	81.3 ± 7.8 *a*	0.61 ± 0.03 *a*
	C + 8.7 nkat GO/g flour	57.5 ± 0.7 *b*	0.31 ± 0.01 *b*
	C + 5.0 μmol H_2O_2/g flour	56.0 ± 7.1 *b*	0.38 ± 0.04 *b*

[a]Values with the same letter are not significantly different ($P < 0.05$) per column.

total FA and total WE-AX concentrations (Table 3). Such a dosage of P_2O or GO resulted in a theoretical production level of 1.3 μmol H_2O_2/g of flour. Under such conditions, only about 30% of the original FA was oxidized, probably because H_2O_2 was a substrate in many other reactions, e.g., in the oxidation of GSH or other SH containing compounds. The decrease in total FA and WE-AX as observed with P_2O or GO was comparable to that when the dough recipe contained 5.0 μmol of H_2O_2/g of flour (Table 3). The WE-AX level decreased by about 48%, whereas that of FA decreased by only about 32%. Probably, the decrease can be explained by a combination of extensive cross-linking of WE-AX and physical incorporation in gluten aggregates, both of which render the WE-AX molecules unextractable.

When adding 0.51 nkat of P_2O or GO/g of flour or 5.0 μmol of H_2O_2/g of flour, the viscosity of dough extract did not significantly differ from that of control dough extract (Table 4).

Table 4. Relative Viscosity and Arabinoxylan (AX, weight % on db) Levels of Extracts of Yeastless Control Dough and Yeastless Dough Supplemented with Pyranose Oxidase (P_2O), Glucose Oxidase (GO), or H_2O_2[a]

sample	relative viscosity	AX (weight % on db)
control (C)	1.20 ± 0.02 *ab*	0.49 ± 0.05 *b*
C + 0.51 nkat P_2O/g flour	1.15 ± 0.03 *b*	0.50 ± 0.03 *b*
C + 0.51 nkat GO/g flour	1.31 ± 0.08 *a*	0.66 ± 0.07 *a*
C + 5.0 μmol H_2O_2/g flour	1.14 ± 0.05 *b*	0.34 ± 0.02 *c*

[a]Relative viscosity values were expressed towards water as a blank. Values with the same letter are not significantly different ($P < 0.05$) per column.

However, the viscosity of dough extract from dough made with P_2O was significantly lower than that of dough extract from dough made with GO (Table 4). Furthermore, AX levels in the dough extract of yeastless control dough and dough made with P_2O were similar, while that of yeastless dough made with GO was significantly higher and that of dough made with H_2O_2 was significantly lower (Table 4). The increase in AX level might be explained by the presence of (cross-linked) AX molecules which are not extracted in the control sample, probably because of incorporation in unextractable compounds. The formed AX cross-links logically increase viscosity. The decrease in AX level can be attributed to extensive cross-linking of AX. Such cross-linking then results in a lower level of AX in dough extract and in a viscosity decrease. These results are in line with those of Vemulapalli and Hoseney[39] and Schooneveld-Bergmans and co-workers.[40] Vemulapalli and Hoseney found that GO does not affect protein extractability in an SDS containing medium but can increase the viscosity of WE.[39] These authors found that the viscosity first increased and then decreased upon the addition of increasing levels of GO. Likewise, Schooneveld-Bergmans and co-workers found that GO increases the viscosity of a solution of wheat flour AX.[40] However, upon continued incubation, the viscosity decreased again. They attributed this decrease to the formation of hydroxyl radicals from H_2O_2, which degrade the polysaccharide structure by radical cleavage, although cross-linking of AX into unextractable AX molecules would seem a more logical explanation. The here observed decrease in viscosity when adding a high enzyme level is, hence, attributed to cross-linking of AX.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

ACN, acetonitrile; AX, arabinoxylan; db, dry matter base; FA, ferulic acid; GO, glucose oxidase; GSH, glutathione; GSSG, oxidized GSH; nkat, nanokatal; P_2O, pyranose oxidase; POD, peroxidase; PU, purpurogallin units; SDS, sodium dodecyl sulfate; SH, sulfhydryl; spm, strokes per minute; SS, disulfide; TFA, trifluoroacetic acid; TYR, tyrosine; WE-AX, water extractable AX

REFERENCES

(1) Veraverbeke, W. S.; Delcour, J. A. Wheat protein composition and properties of wheat glutenin in relation to breadmaking functionality. *Crit. Rev. Food Sci.* **2002**, *42*, 179−208.

(2) Zghal, M. C.; Scanlon, M. G.; Sapirstein, H. D. Effects of flour strength, baking absorption, and processing conditions on the structure and mechanical properties of bread crumb. *Cereal Chem.* **2001**, *78*, 1−7.

(3) Lagrain, B.; Brijs, K.; Delcour, J. A. Impact of redox agents on the physico-chemistry of wheat gluten proteins during hydrothermal treatment. *J. Cereal Sci.* **2006**, *44*, 49−53.

(4) Bonet, A.; Rosell, C. M.; Caballero, P. A.; Gomez, M.; Perez-Munuera, I.; Lluch, M. A. Glucose oxidase effect on dough rheology and bread quality: A study from macroscopic to molecular level. *Food Chem.* **2006**, *99*, 408−415.

(5) Tilley, K. A.; Benjamin, R. E.; Bagorogoza, K. E.; Okot-Kotber, B. M.; Prakash, O.; Kwen, H. Tyrosine cross-links: Molecular basis of gluten structure and function. *J. Agric. Food Chem.* **2001**, *49*, 2627−2632.
(6) Hanft, F.; Koehler, P. Quantitation of dityrosine in wheat flour and dough by liquid chromatography-tandem mass spectrometry. *J. Agric. Food Chem.* **2005**, *53*, 2418−2423.
(7) Pena, E.; Bernardo, A.; Soler, C.; Jouve, N. Do tyrosine crosslinks contribute to the formation of the gluten network in common wheat (*Triticum aestivum* L.) dough? *J. Cereal Sci.* **2006**, *44*, 144−153.
(8) Courtin, C. M.; Delcour, J. A. Arabinoxylans and endoxylanases in wheat flour bread-making. *J. Cereal Sci.* **2002**, *35*, 225−243.
(9) Izydorczyk, M. S.; Biliaderis, C. G. Cereal arabinoxylans: Advances in structure and physicochemical properties. *Carbohydr. Polym.* **1995**, *28*, 33−48.
(10) Hilhorst, R.; Dunnewind, B.; Orsel, R.; Stegeman, P.; van Vliet, T.; Gruppen, H.; Schols, H. A. Baking performance, rheology, and chemical composition of wheat dough and gluten affected by xylanase and oxidative enzymes. *J. Food Sci.* **1999**, *64*, 808−813.
(11) Labat, E.; Morel, M. H.; Rouau, X. Effect of laccase and manganese peroxidase on wheat gluten and pentosans during mixing. *Food Hydrocolloids* **2001**, *15*, 47−52.
(12) Wang, M. W.; Hamer, R. J.; van Vliet, T.; Oudgenoeg, G. Interaction of water extractable pentosans with gluten protein: Effect on dough properties and gluten quality. *J. Cereal Sci.* **2002**, *36*, 25−37.
(13) Piber, M.; Koehler, P. Identification of dehydro-ferulic acid-tyrosine in rye and wheat: Evidence for a covalent cross-link between arabinoxylans and proteins. *J. Agric. Food Chem.* **2005**, *53*, 5276−5284.
(14) Decamps, K.; Joye, I. J.; Courtin, C. M.; Delcour, J. A. Glucose and pyranose oxidase improve bread dough stability. *J. Cereal Sci.* **2012**, *55*, 380−384.
(15) Bonet, A.; Rosell, C. M.; Perez-Munuera, I.; Hernando, I. Rebuilding gluten network of damaged wheat by means of glucose oxidase treatment. *J. Sci. Food Agric.* **2007**, *87*, 1301−1307.
(16) Dunnewind, B.; van Vliet, T.; Orsel, R. Effect of oxidative enzymes on bulk rheological properties of wheat flour doughs. *J. Cereal Sci.* **2002**, *36*, 357−366.
(17) Steffolani, M. E.; Ribotta, P. D.; Perez, G. T.; Leon, A. E. Effect of glucose oxidase, transglutaminase, and pentosanase on wheat proteins: Relationship with dough properties and bread-making quality. *J. Cereal Sci.* **2010**, *51*, 366−373.
(18) Primo-Martin, C.; Wang, M. W.; Lichtendonk, W. J.; Plijter, J. J.; Hamer, R. J. An explanation for the combined effect of xylanase-glucose oxidase in dough systems. *J. Sci. Food Agric.* **2005**, *85*, 1186−1196.
(19) Decamps, K.; Joye, I. J.; Haltrich, D.; Nicolas, J.; Courtin, C. M.; Delcour, J. A. Biochemical characteristics of *Trametes multicolor* pyranose oxidase and *Aspergillus niger* glucose oxidase and implications for their functionality in wheat flour doughs. *Food Chem.* **2012**, *131*, 1485−1492.
(20) *Approved Methods of the AACC*, 11th ed.; AACC International: St. Paul, MN, 2012.
(21) *Official Methods of Analysis*; AOAC: Washington, DC, 1995.
(22) Ellman, G. L. Tissue sulfhydryl groups. *Arch. Biochem. Biophys.* **1959**, *82*, 70−77.
(23) Rakotozafy, L.; Buisson, D.; Levavasseur, L.; Verté, F.; Potus, J.; Nicolas, J. Oxidation of tyrosine by laccase and peroxidases in presence of thiol compounds. Poster presented at 5th European Symposium on Enzymes in Grain Processing (ESEGP5), Norwich, U. K., 2008.
(24) Hoseney, R. C.; Hsu, K. H.; Junge, R. C. Simple spread test to measure the rheological properties of fermenting dough. *Cereal Chem.* **1979**, *56*, 141−143.
(25) Courtin, C. M.; Gelders, G. G.; Delcour, J. A. Use of two endoxylanases with different substrate selectivity for understanding arabinoxylan functionality in wheat flour breadmaking. *Cereal Chem.* **2001**, *78*, 564−571.
(26) Lagrain, B.; Brijs, K.; Veraverbeke, W. S.; Delcour, J. A. The impact of heating and cooling on the physico-chemical properties of wheat gluten-water suspensions. *J. Cereal Sci.* **2005**, *42*, 327−333.
(27) Veraverbeke, W. S.; Larroque, O. R.; Bekes, F.; Delcour, J. A. In vitro polymerization of wheat glutenin subunits with inorganic oxidizing agents. I. Comparison of single-step and stepwise oxidations of high molecular weight glutenin subunits. *Cereal Chem.* **2000**, *77*, 582−588.
(28) Dobberstein, D.; Bunzel, M. Separation and detection of cell wall-bound ferulic acid dehydrodimers and dehydrotrimers in cereals and other plant materials by reversed phase high-performance liquid chromatography with ultraviolet detection. *J. Agric. Food Chem.* **2010**, *58*, 8927−8935.
(29) Courtin, C. M.; Van den Broeck, H.; Delcour, J. A. Determination of reducing end sugar residues in oligo- and polysaccharides by gas-liquid chromatography. *J. Chromatogr., A* **2000**, *866*, 97−104.
(30) Englyst, H. N.; Cummings, J. H. Simplified method for the measurement of total non-starch polysaccharides by gas-liquid-chromatography of constituent sugars as alditol acetates. *Analyst* **1984**, *109*, 937−942.
(31) Van den Bulck, K.; Swennen, K.; Loosveld, A. M. A.; Courtin, C. M.; Brijs, K.; Proost, P.; Van Damme, J.; Van Campenhout, S.; Mort, A.; Delcour, J. A. Isolation of cereal arabinogalactan-peptides and structural comparison of their carbohydrate and peptide moieties. *J. Cereal Sci.* **2005**, *41*, 59−67.
(32) Garcia, R.; Kaid, N.; Vignaud, C.; Nicolas, J. Purification and some properties of catalase from wheat germ (*Triticum aestivum L.*). *J. Agric. Food Chem.* **2000**, *48*, 1050−1057.
(33) Garcia, R.; Rakotozafy, L.; Telef, N.; Potus, J.; Nicolas, J. Oxidation of ferulic acid or arabinose-esterified ferulic acid by wheat germ peroxidase. *J. Agric. Food Chem.* **2002**, *50*, 3290−3298.
(34) Louarme, L. *Etude du mécanisme d'action du bromate de potassium en panification en vue de le remplacer par une oxydoréductase (Study of the potassium bromate effect in breadmaking in order to replace it by an oxidoreductase)*; PhD Thesis, University of Paris-Sud: Paris, France, 2001.
(35) Hird, F. J.; Yates, J. R. Oxidation of cysteine, glutathione and thioglycollate by iodate, bromate, persulphate and air. *J. Sci. Food Agric.* **1961**, *12*, 89−95.
(36) Shewry, P. R.; Popineau, Y.; Lafiandra, D.; Belton, P. Wheat glutenin subunits and dough elasticity: Findings of the Eurowheat project. *Trends Food Sci. Technol.* **2001**, *11*, 433−441.
(37) Veraverbeke, W. S.; Courtin, C. M.; Verbruggen, I. M.; Delcour, J. A. Factors governing levels and composition of the sodium dodecyl sulphate-unextractable glutenin polymers during straight dough breadmaking. *J. Cereal Sci.* **1999**, *29*, 129−138.
(38) Rasiah, I. A.; Sutton, K. H.; Low, F. L.; Lin, H. M.; Gerrard, J. A. Crosslinking of wheat dough proteins by glucose oxidase and the resulting effects on bread and croissants. *Food Chem.* **2005**, *89*, 325−332.
(39) Vemulapalli, V.; Hoseney, R. C. Glucose oxidase effects on gluten and water solubles. *Cereal Chem.* **1998**, *75*, 859−862.
(40) Schooneveld-Bergmans, M. E. F.; Dignum, M. J. W.; Grabber, J. H.; Beldman, G.; Voragen, A. G. J. Studies on the oxidative cross-linking of feruloylated arabinoxylans from wheat flour and wheat bran. *Carbohydr. Polym.* **1999**, *38*, 309−317.