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Introduction.

Addition of xylanases has been shown to have a beneficial effect on dough properties and bread volume (Rouau et al., 1994, Sprössler, 1995, Poutanen, 1997). This is thought to be caused by the conversion of insoluble arabinoxylans into soluble ones, which causes changes in the distribution of water in dough (Maat et al, 1992). Pentosans are known to have a high water binding capacity, the soluble ones more than the insoluble ones (Krishnarau and Hoseney, 1994). An alternative explanation for the positive effect of xylanase is that arabinoxylans interfere with the formation of a gluten network, and their removal allows the formation of a stronger, more elastic network (Weegels, 1990, van der Lugt et al, 1995). However, depending on the source of xylanase and fermentation conditions, the dough may become sticky and difficult to handle. Addition of peroxidase has been shown to counteract this (van Oort et al, 1995a, 1995b, van Oort, 1996). It has been suggested (van Oort, 1996) that peroxidase exerts its effect by cross-linking arabinoxylans via ferulic acid residues that are an integral part of this polymer. In vitro, such cross-linked arabinoxylans are able to form gels with a large water holding capacity (Geissman, 1973). Since peroxidases are also able to cross-link proteins via tyrosine residues (Sizer and Gross,1959), coupling to proteins or protein-protein coupling has also been suggested (Neukom, 1976, Matheis and Whitaker 1984, 1987).

Materials and Methods.

Dough preparation.

The flour used was a commercial untreated cookie type flour Kolibri with 15.0% moisture and 10.5% protein, obtained from Meneba Meel B.V. Rotterdam, The Netherlands. Inherently, a cookie type flour has a poor bread making quality. Yeast was "Koningsgist" from Gist-brocades, Delft, The Netherlands.

Fungal α-amylase (Biobake 5000, 175 FFA/mg), xylanase ex *Trichoderma* (Biobake CX160, 351 U/mg measured on oat spelt xylan) (XYL) and soy peroxidase (Biobake soy, 35 U/mg) (POX) were from Quest International BV, Naarden, The Netherlands.

Dough's were prepared with 2000 g Kolibri flour as is, 1180 g water, 40 g NaCl, 80 g yeast, 0.04 g ascorbic acid and 0.1 g α-amylase. To XYL containing dough's 0.6 g xylanase was added. POX containing dough's were supplemented with 4.4 mg peroxidase. Dough's were mixed in a Kemper SP 15 spiral mixer (Neuenkircher Eisengiesserei und Machinenfabrik, Rietberg, Germany), at 300 slow turns and 800 fast turns, resulting in a dough temperature of 26 °C. Dough's were rested for 30 minutes at 26 °C and either used for baking trials, for gluten isolation by hand washing (15 min) with water containing 2 % sodium chloride or frozen at -20 °C in portions of 500 g.

Baking trials.
For crispy rolls, after 15 min of first proofing, the dough was divided into 55 g pieces, using a Record Automat type 3-10 TLG, from Kemper. The dough pieces were shaped using an Frilado type 2 shaper (Fritz Laureck). After 50 or 65 min proofing they were baked in a Winkler Wachtel 4-Deck Oven type AE 416/38 for 19 min at 220 °C. White tin breads were baked using the same process conditions, but the dough was divided after 5 min of first proofing and baked after 55 or 70 min final proofing.
The dough's were scored manually (rheological techniques cannot be applied to yeasted doughs) two minutes after mixing for dryness, stiffness, elasticity, extensibility and coherency on a 10 point scale. Scoring was done blindly by 3 skilled bakers, each having more than 5 years experience, per set of 3 dough's including a reference dough. Dryness was determined by touching the dough; 1 was extremely sticky, 10 very dry. Stiffness (firmness) was the resistance of the dough when applying manual pressure; 1 was very soft, 10 was very stiff. Elasticity was the capability of the dough to regain its original shape after being extended; 1 was non-elastic (deformation not recoverable), 10 was very elastic, (deformation totally recoverable). Extensibility was the deformation to fracture when extended manually with a speed of about 5 cm/s; 1 was not extensible, 10 was very extensible. Coherency was measured as the sheet forming capacity; 1 was non-coherent, 10 was very coherent.
The fermentation stability of the crispy rolls was scored manually after the standard proofing time of 50 min and at 65 min proofing time (15 min over-proofing). The baked crispy rolls were scored by experienced test bakers on a 10 point scale for roll shape and crumb structure; 1 was a very coarse structure, 10 was small cells and very regular structure. Product volume was measured using the rape seed displacement method. Tin loaves were judged for stability, ovenspring, crumb structure and softness.

Fractionation of dough
After thawing of the dough overnight at 4 °C, it was fractionated into seven fractions according to Scheme 1 as described by Gruppen et al. (1989) with minor modifications. Crude WUS1 and crude WUS 2 were extracted at 95 °C instead of 63 °C, in order to inactivate enzymes present in those fractions. After concentration, CWE, HWE1, HWE2 were dialysed against distilled water prior to freeze drying and subsequent grinding.

Rheological measurements.
Biaxial extension tests were performed with fresh gluten by compression of a cylindrical test piece of gluten between two parallel Teflon plates with radius R = 10 mm, lubricated with paraffin oil (110 mPa·s) (Van Vliet et al., 1992). A gluten piece of 6.0 g was placed in a lubricated Teflon cylinder with diam 19 mm and covered with a lubricated solid Teflon plunger. After 45 min the sample was compressed in a Lloyd instruments material testing machine (LR5K) equipped with a 50 N load cell, at room temperature (27 °C). At least 18 test pieces were compressed at 3 speeds v (5, 12 and 60 mm/min) to a final height of 1 mm. The deformation and force F were recorded. The point at which a force of 0.01 N was reached was taken as the starting point of compression. Hencky strain ε_H and strain rate $\dot{\varepsilon}$ were calculated as $\varepsilon_H = -\ln(h_t/h_0)$ and $\dot{\varepsilon} = d\varepsilon_H/dt = dh/(hdt) = v/h_t$, respectively; biaxial strain and biaxial extensional rate as $\varepsilon_B = \varepsilon_H/2$ and $\dot{\varepsilon}_B = \dot{\varepsilon}_H/2 = v/2h_t$, respectively; h_0 is the initial height of the test piece and h_t the height at time t. Stress was calculated as $\sigma = F_t/(\pi R^2)$ (Van Vliet et al., 1992; Van Vliet, 1998).

Analytical techniques.
The protein content was determined by a semi micro Kjeldahl method and calculated from the nitrogen content (N*5.75) (Gruppen et al., 1989).
Starch was determined enzymatically with a testkit (Boehringer Mannheim)
Prior to the determination of neutral sugars, gluten was extracted with DMSO/H_2O/ethanol (4:16:80 v/v) to remove (galacto)lipids (Gruppen et al., 1989). The neutral sugars in the gluten

were determined as alditol acetates using inositol as internal standard (Englyst and Cummings, 1984). Hydrolysis was performed with 1 M H_2SO_4 for 3 h at 100 °C, so cellulosic glucose was not determined (Englyst et al, 1982).
Alditol acetates were separated on a 2 m * 2 mm i.d. glass column (packed with Chrom WAW 80-100 mesh coated with 3 % OV275) in a Carlo Erba Fractovap 2300 GC operated at 200 °C and equipped with a F.I.D. detector set at 270 °C. Non-starch glucose was calculated from the difference in glucose found by the alditol acetate method and by the starch determination.
Phenolic acid content was determined as described by Gruppen et al., (1989). The samples were analyzed by HPLC with a reversed phase Lichrosorb 10 RP 18 column (Merck; 250*4.6 mm), at room temperature, at a flow rate of 1 mL/min. Column elution conditions were modified: elution started with 95 % 0.01 M acetic acid pH 5.0 and 5% methanol for 5 min. The methanol concentration increased to 50 % in 20 min, to 90 % in 5 min, and subsequently to 95 % in 5 min. After 10 min at 95 % methanol, the initial conditions were established over a period of 1 min, followed by 14 min washing. The eluent was monitored from 200 to 360 nm with a Spectra System UV3000 rapid scanning UV/VIS detector (Thermo Separation Products). Phenolic acid concentrations were calculated with respect to p-hydroxybenzoate as internal standard, and corrected for the response factors of coumaric and ferulic acid at 280 nm.
High performance size-exclusion chromatography (HPSEC) was performed as described by Oosterveld et al (1997) using Biorad Biogel TSK 60XL, 40 XL and 30 XL columns in series in combination with a TSK XL guard column on a Spectra Physics 8700 XR system HPLC. Elution was carried out with 0.4 M sodium acetate buffer pH 3.0 at a flow rate of 0.8 ml/min at 30 °C. The eluate was monitored with a combined RI detector and viscometer (Viscotek, model 250), a Right Angle Laser Light-Scattering detector (RALLS, Viscotek, LD600) and a UV detector (Kratos, Spectroflow 773) set at 280 and 320 nm. Molecular weights were calculated using the light-scattering and universal calibration modules of the Trisec software (Viscotek). Dextran standards were from Amersham Pharmacia.

Release of cross-links.
Starch was removed from CWE, HWE1, HWE2, WUS1 and WUS2 by incubation with α-amylase and pullulanase (both from Boehringer, Mannheim, Germany) overnight at 30 °C in 20 % DMSO in 0.1 M sodium acetate pH 5.0, followed by dialysis against distilled water and freeze drying. Each fraction underwent three treatments: control (5 mg /ml was incubated overnight at room temperature with 0.1 M sodium acetate buffer pH 6.5), alkali (10 mg/ml was incubated overnight at room temperature in 0.5 N KOH) or protease (5 mg/ml was incubated overnight at room temperature with trypsin (Sigma) (0.005 mg /mg) in 0.1 M sodium acetate, pH 6.5). After neutralisation with an equal volume acetic acid (KOH incubation) or inactivation in a boiling water bath for 5 min (trypsin incubation), the insoluble material was precipitated by centrifugation. In the supernatants, the size distribution, the protein and ferulic acid content and the sugar composition were determined by HPSEC as described above.

Statistical analysis.
All determinations were performed in duplicate. Reproducibility was generally better than 2 %. Data were analysed using the Statistical Analysis System (SAS Institute, Cary, NC). Significantly different means were separated using least significant difference.

Results and Discussion.

Baking performance.
Dough's prepared without xylanase (control), with xylanase (XYL), with peroxidase (POX) and with xylanase and peroxidase (XYL/POX) were judged for their properties by a panel of experienced bakers. The results are given in Table 1, together with the properties of the fermenting dough's and the baked products. Addition of xylanase resulted indeed in a weak

dough. The adverse effect of xylanase was counteracted by POX, resulting is a dough with good handling properties. Addition of POX alone gave a rather stiff and poorly extensible dough. Note that the effect of these enzymes was already noticeable 2 min after mixing. The combination XYL/POX also gave the best results with respect to fermentation stability and properties of the baked product.

Table 1. Evaluation of manually scored dough properties 2 min after mixing and baking performance of the different dough's at 50 min proofing time or 15 min overproofing time (65 min).

Dough properties		**Control**	**XYL**	**POX**	**XYL/POX**
Dryness		5	4	7	7
Stiffness		6	4	8	7
Elasticity		4	5	6	6
Extensibility		3	4	2	4
Coherency		5	6	7	7
Properties of fermenting dough and baked rolls.					
	Proofing time				
Fermentation stability	50	6	7	7	8
	65	4	6	6	7
Specific volume (%)	50	100	112	102	114
	65	100	116	104	120
Shape of roll	50	5	6	6	7
	65	4	5	5	6
Crumb structure	50	5	7	6	7
	65	3	6	5	7

Effect of xylanase and peroxidase on gluten.

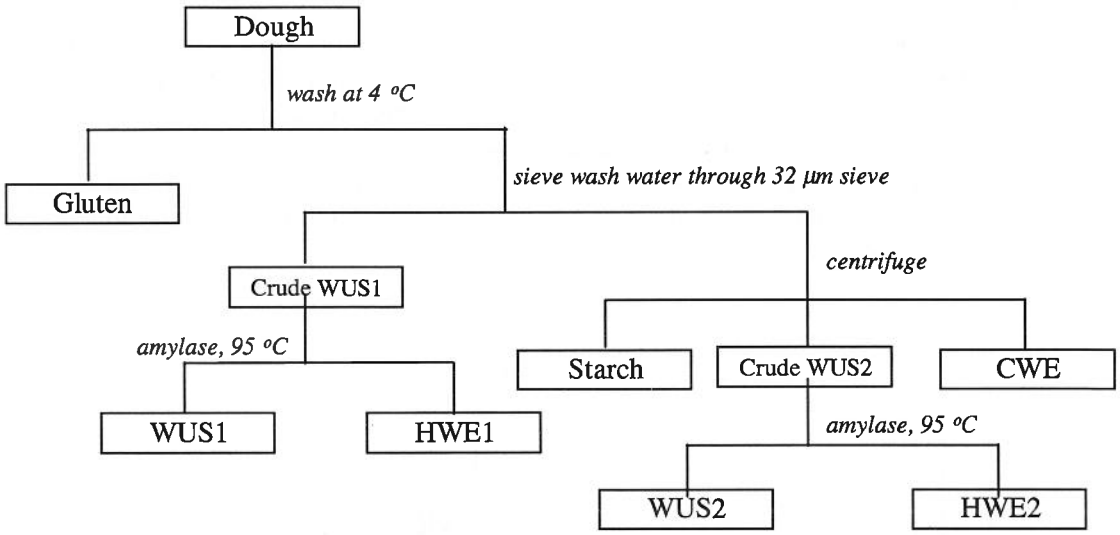
To investigate whether xylanase and peroxidase influenced the properties of the gluten, gluten were isolated from dough's after 30 min of fermentation, and subjected to rheological investigations and chemical analyses. The stress of the fresh gluten as a function of the biaxial strain showed slight variations. From these curves strain hardening values were calculated. This yielded a strain hardening value of 2.2 for control gluten. Addition of XYL lowered the value to 2.0, but for XYL/POX gluten a value of 2.4 was obtained, higher than the value for POX alone (2.3) (Hilhorst et al, 1999a). Stress and strain hardening are important factors for bread crumb structure. Stress is most important at the beginning of proofing, while strain hardening is important at the end. Higher stress favours good crumb structure, and strain hardening is an important factor for dough film stability. The higher the strain hardening, the better the stability against rupture of dough films between gas cells (van Vliet, 1998). The crumb structure of the baked product can be related to dough fracture. During fermentation, the gas cell increase in volume, and the liquid dough films between the gas cells are extended. When the extension becomes too large, the films fracture and the gas cells coalesce and lower the gas holding capacity of dough. The biaxial extension tests show that the dough prepared with peroxidase and xylanase was more extensible because of synergistic effects of the enzymes on gluten stress and strain hardening. The increased extensibility and stronger strain hardening may explain the finer crumb structure of the XYL/POX loaves.

The rheological data did not provide any evidence that xylanase removed polysaccharides from the gluten, leading to a better aggregation of the gluten, as suggested by van der Lugt et al. (1995). In that case xylanase should have caused a higher stress and stronger strain hardening, except if it also caused the gluten network to become much less homogeneous, which is less likely.

The amounts of starch, protein, sugars and ferulic acid in the gluten fractions were determined. The gluten from control dough contained more starch, more non-starch polysaccharides and more arabinose and xylose than the other three glutens, but less protein.

This can be attributed to less efficient washing (Saulnier et al, 1997). Both for the control and the other glutens, the amount of arabinoxylans was 50 % of the total amount of non-starch polysaccharides. Therefore no evidence for specific removal of arabinoxylans from gluten by xylanase was obtained. Furthermore, since the POX glutens contained the same amount of arabinoxylans as the xylanase glutens, POX does not cross-link arabinoxylans to gluten.
The properties of the gluten per se do not suffice to obtain a good quality dough and a good baking performance. Properties of other fractions of the dough are also relevant for the performance.

Effect of peroxidase on non-starch polysaccharides.
To investigate the effect of xylanase and peroxidase on other dough fractions, dough's prepared with XYL and XYL/POX were fractionated according to scheme 1. This resulted in four insoluble fractions: Gluten, Starch, large cell wall fragments (WUS1) and small cell wall fragments (WUS2). The soluble fractions were CWE (Cold Water Extract), HWE1 and HWE2, derived by hot water extraction from the large cell wall fragments (HWE1) and from the small cell wall fragments (HWE2). These fractions were dialysed to remove low molecular weight material.



Scheme 1. Fractionation of dough.

The composition of the individual fractions was determined. Addition of POX resulted in more protein, more non-starch polysaccharides and more ferulic acid, notably in the insoluble fractions. The composition of gluten from both dough's however, was not significantly different, in agreement with the previous results. Analysis of the sugar composition revealed that the amounts of arabinose and xylose had increased substantially in fractions from XYL/POX dough.

Table 2. Yield and composition of the fractions isolated from 500 g dough with XYL or XYL/POX.

	weight (g)		starch (g)		protein (g)		NSP (g)		ferulic acid (mg)	
Fraction	XYL	XYL/ POX	XYL	XYL/ POX	XYL	XYL/ POX	XYL	XYL/ POX	XYL	XYL/ POX
Insoluble	193.9	201.4	161.5	162.5	21.4	22.7	3.0	4.3	15.1	17.3
Soluble	14.9	12.8	4.2	3.1	4.4	4.7	1.7	1.7	4.1	3.5
Total	208.8	214.2	165.7	165.6	25.8	27.4	4.7	6.0	19.2	20.8

NSP: Non-starch polysaccharides.

The major differences were found between the fractions WUS2 and HWE2. Whereas the first one had increased in weight due to peroxidase action, the weight of the second one was reduced. The amounts of protein, NSP and ferulic acid in WUS2 had increased considerably. The increase in NSP could be attributed to a higher content in ara and xy. The ara/xy ratio was 0.83 in XYL WUS2 and 0.63 in XYL/POX WUS2, indicative of the addition of arabinoxylans with a low degrees of substitution. Such arabinoxylans are present in CWE and HWE2. This is in agreement with the notion that peroxidase acts by cross-linking arabinoxylans, rendering them insoluble.

It is assumed that peroxidase cross-links via ferulic acids. Ferulic acids are linked to arabinose via ester linkages, that are hydrolysed under alkaline conditions. Therefore, incubation with alkali would release arabinoxylans cross-linked via ferulic acids. Linkages in the arabinoxylan backbone are not susceptible to this treatment. If, on the other hand, arabinoxylans have been linked to proteins, incubation with protease would release arabinoxylans with small protein fragments attached.
Supernatants of WUS2 from XYL and XYL/POX dough have been analysed for protein, sugar and ferulic acid content after incubation with buffer, alkali or protease. Fig. 1A shows that alkali treatment released a large amount of protein, arabinose and xylose from XYL WUS2. Protease treatment solubilised only protein. Alkali treatment not only liberated arabinose and xylose from XYL/POX WUS2, but also large amounts of protein (Fig. 1B). Protease treatment too released protein, and some arabinose and xylose. The ara/xy rations of the solubilised arabinoxylans were 0.63 and 0.66 respectively for alkali and protease treatment, whereas the ara/xy ratio of arabinoxylans released from XYL WUS2 was 0.94. This again points at the addition of less substituted arabinoxylans to WUS2, due to peroxidase action.

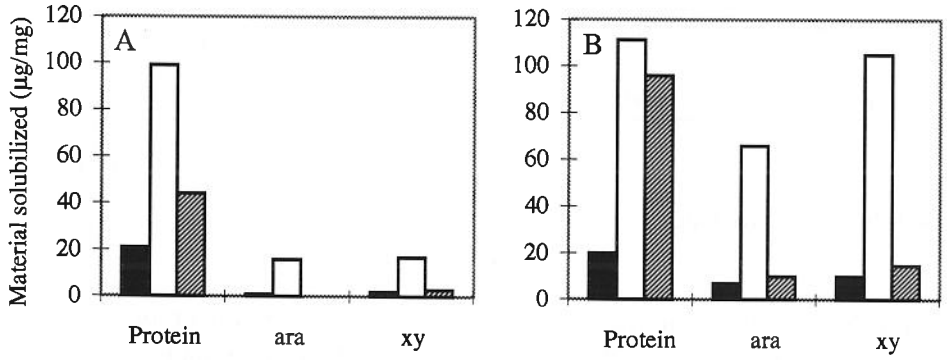


Fig. 1. Amount of protein, arabinose (ara) and xylose (xy) solubilized from WUS2 from XYL dough (A) or from XYL/POX dough (B) after treatment with buffer (grey bar), alkali (white bar) or protease (shaded bar).

The size distribution of carbohydrates was investigated with HPSEC. WUS2 from XYL dough contained carbohydrates with molecular weights in the range of 40 kDa, whereas those liberated from WUS2 from XYL/POX dough had molecular weights above 1000 kDa. Protease treatment liberated only very few carbohydrates. On the basis of the elution pattern monitored at 280 and 320 nm (protein and ferulic acid absorption respectively) is must be concluded that at most a small amount of arabinoxylan has been covalently coupled to protein.

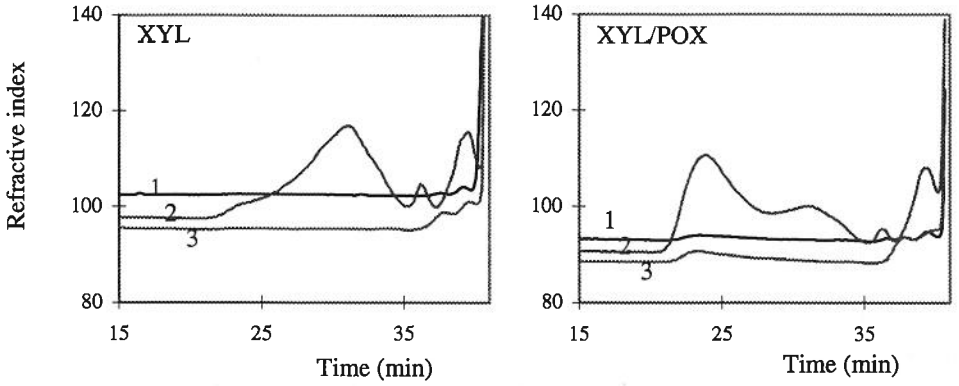


Fig. 2. HPSEC elution patterns with refractive index detection of WUS2 from XYL and XYL/POX dough after incubation with sodium acetate buffer (1), alkali (2) or protease (3).

This is in agreement with *in vitro* studies where addition of protein did not influence the rate of gelling of arabinoxylans (Schooneveld-Bergmans et al., 1998, Figueroa–Espinoza and Rouau, 1998) and with studies in our group (Oudgenoeg et al., unpublished results), that indicate that only under specific optimised conditions peroxidase can couple ferulic acids to tyrosines.

Conclusions.

Addition of xylanase and peroxidase was shown to have a positive effect in bread making. The combination XYL/POX gave the best handling properties for wheat bread dough's and also a baked product with a good crumb structure, high volume and good fermentation stability.
The results presented here exclude some of the mechanisms of action that have been suggested for xylanase and peroxidase. Both rheology and chemical analyses showed that gluten properties were not significantly affected. Peroxidase was shown to act by cross-linking arabinoxylans via ferulic acid to other arabinoxylans. No cross-linking to gluten proteins occured, arabinoxylans were mainly attached to WUS2.

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