

# Use of Two Endoxylanases with Different Substrate Selectivity for Understanding Arabinoxylan Functionality in Wheat Flour Breadmaking

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## ABSTRACT

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A *Bacillus subtilis* endoxylanase (XBS) with a strong selectivity for hydrolysis of water-unextractable arabinoxylan (WU-AX) and an *Aspergillus aculeatus* endoxylanase (XAA) with a strong selectivity for hydrolysis of water-extractable arabinoxylan (WE-AX) were used in straight-dough breadmaking with two European wheat flours. Dough, fermented dough, and bread characteristics with different levels of enzyme addition were evaluated with a strong emphasis on the arabinoxylan (AX) population. The WU-AX solubilized by XBS during breadmaking were mainly released

during mixing and had higher molecular weight, in contrast to their counterparts solubilized by XAA, which were mainly released during fermentation and had lower molecular weight. This coincided with increased loaf volume with XBS and a negative to positive loaf volume response with XAA. Bread firmness and dough extract viscosity also were affected by endoxylanase addition. Results confirmed that WU-AX are detrimental for breadmaking, while WE-AX and solubilized AX with medium to high molecular weight have a positive impact on loaf volume.

Endo- $\beta$ -1,4-xylanases (EC 3.2.1.8.), also referred to as endoxylanases or xylanases, find widespread use in breadmaking applications. In combination with emulsifiers, oxidizing agents, and other enzymes such as lipases, lipoxygenases, glucose, or hexose oxidases, and amylases, they are often added to a variety of bread-improver mixtures. Despite factual acceptance of endoxylanases as a means of improving dough and bread characteristics, their mode of action is still the subject of debate (Maat et al 1992; Rouau et al 1994; Popper 1997; Sprössler 1997; Hillhorst et al 1999).

Endoxylanase functionality in breadmaking is, evidently, the result of the enzymic modification of the arabinoxylan (AX) population in dough and bread. Water-unextractable AX (WU-AX), which make up 70% of wheat endosperm cell walls (Mares and Stone 1973), can be hydrolyzed and made soluble, which causes them to lose their strong water-holding capacity (Gruppen et al 1993). Solubilization of WU-AX can be responsible for an increase in viscosity in the dough aqueous phase. Degradation of enzyme-solubilized AX (ES-AX) and native water-extractable AX (WE-AX) results in a decrease in molecular weight of AX fragments in solution and a decrease in their viscosity-forming properties (Rouau and Moreau 1993; Petit-Benvegnen et al 1998).

The effect of each of the above-cited endoxylanase activities on baking absorption, loaf volume, and rheology of wheat flour doughs was studied separately by fractionation-reconstitution experiments (Courtin et al 1999). The results suggested that endoxylanases do not increase loaf volume by dynamic release of water from the WU-AX in favor of the gluten (Maat et al 1992), but do so by removing the deleterious WU-AX fraction and by increasing the level of medium to high molecular weight ES-AX in the water phase. The results further confirmed the positive correlation between WU-AX quantity and WE-AX molecular weight on the one hand and baking absorption on the other hand (Kulp 1968; Courtin and Delcour 1998).

Based on these fractionation-reconstitution experiments, which involved *in vitro* modification of flour fractions by endoxylanase rather than *in situ* modification in the different phases of the breadmaking process (Courtin et al 1999), and on literature data (Rouau et al 1994; Popper 1997), the hypothesis was put forward that an endoxylanase that preferentially hydrolyzes the WU-AX and leaves the WE-AX and ES-AX unharmed would have beneficial effects on bread loaf volume. An endoxylanase with a preference for WE-AX

and ES-AX that only minimally hydrolyzes WU-AX would have little or no effect.

To test this hypothesis in industrial breadmaking conditions (where the endoxylanases are present in the breadmaking recipe) and to assess the impact of different changes in the AX population on dough and bread parameters, two endoxylanases with widely different substrate selectivity were sought. They were identified through a method that was developed to define the relative activity of endoxylanases toward WE-AX and WU-AX (Courtin and Delcour 2001). In this article, we report on the use of these enzymes on two commercially available breadmaking flours at six different levels of enzyme addition. A strong emphasis is placed on the characterization of the AX population in relation to overall dough and bread characteristics.

## MATERIALS AND METHODS

### Materials

A *Bacillus subtilis* endoxylanase (XBS, EC 3.2.1.8), free from  $\alpha$ - and  $\beta$ -amylase,  $\beta$ -xylosidase,  $\beta$ -glucanase, and protease side activities was obtained from Puratos NV (Groot-Bijgaarden, Belgium). Shearzyme 500L, a monocomponent endoxylanase preparation from *Aspergillus aculeatus* (XAA), was obtained from Novo Nordisk (Bagsvaerd, Denmark). A detailed description of properties and activities (Courtin and Delcour 2001) and of sensitivity toward inhibition by endoxylanase inhibitors (Gebruers et al 2001) of both enzymes is given elsewhere. The enzyme activity determined for native WE-AX was 5,230 nKat/mL for XBS and 5,680 nKat/mL for XAA.

All reagents were of at least analytical grade and supplied by Sigma-Aldrich (Bornem, Belgium) unless specified otherwise. Standard P-82 pullulans were purchased from Showa Denko K.K. (Tokyo). Azurine crosslinked arabinoxylan tablets (AZCL-AX) were obtained from Megazyme (Bray, Ireland).

### Flours

Standaard B (flour-A) and Nachtegaal (flour-B) are commercial European wheat flours, free from additives, from Paniflower (Antwerpen, Belgium) and Meneba Meel NV (Rotterdam, The Netherlands), respectively. Protein contents (% dm, N  $\times$  5.7) were 11.6 and 11.7% and ash contents (% dm) were 0.50 and 0.47% for flour-A and flour-B, respectively, as determined by the suppliers.

### Breadmaking Process and Dough and Bread Sampling

Flour-A was processed into bread at Vamix NV (Gent, Belgium) by an experienced baker according to a straight-dough breadmaking procedure. The recipe was flour (8.0 kg), yeast (2.0%), salt (2.0%), sugar (2.5%), and ascorbic acid (20 ppm) mixed with water in an

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arthofex mixer for 15 min. Enzyme concentrations were 0–2.08 nKat/g of flour for XBS and 0–6.00 nKat/g of flour for XAA. Baking absorption was evaluated by an experienced baker. Farinograph (Brabender, Duisburg, Germany) consistency was determined by transferring 480 g of dough into a 300-g flour farinograph bowl immediately after mixing. After a bulk rise of 15 min, the dough was divided into pieces (950 g) and rounded by hand. Dough pieces were allowed to rise for another 15 min before mechanical molding, panning, and proofing (65 min). Doughs were baked at 225°C for 45 min. Steam was applied plentifully at the beginning. Two hours after baking, loaf volumes were measured through rapeseed displacement. Breads were stored in paper bags at 25°C until further analysis.

Flour-B, with different characteristics, was processed at Meneba Meel NV in a similar procedure and with another experienced baker. Flour (3.5 kg) with yeast (2.0%), salt (2.0%), sugar (2.5%), and ascorbic acid (15 ppm) were mixed with water in a spiral mixer for 3 min at low speed and 2.5 min at high speed. Baking absorption again was manually evaluated. Bulk rise was 50 min and dough pieces were weighed (490 g, dm). Mechanical rounding was followed by fermentation (35 min), mechanical molding, panning, and proofing (70 min). Doughs were transferred to an oven (340°C), which was then switched off. Steam was applied and bread was baked for 35 min. After 2 hr, loaf volume was measured by rapeseed displacement. Breads were sealed in plastic bags and stored at 25°C. The coefficient of variation (CV) for specific loaf volume was <2.2% for breadmaking trials with either flour-A or B.

Dough samples taken immediately after kneading and proofing and bread samples withdrawn 24 hr after baking were frozen in liquid nitrogen. After lyophilization, they were ground and sieved ( $\varnothing = 250 \mu\text{m}$ ).

### Dough and Bread Scoring

An overall, subjective assessment of dough and bread characteristics was given by both bakers for each flour and enzyme combination. This assessment was converted into three scores: a dough score, a processing score, and a bread score, each with a range of 1–5, where a higher score represented better characteristics. The dough score consisted of a combination of several dough parameters such as elasticity and stickiness. A dough score of 1 represented an unmanageable dough, while a dough score of 5 was given to dough with optimal characteristics. A processing score was attributed to the doughs in the same manner and reflected the overall manageability of the dough during the remainder of the bread-making process. Here, softening, stickiness, and flowing of the dough were the main characteristics evaluated. Doughs exhibiting these characteristics were given a low score of 1, while doughs that kept their form, were not too soft, and had good machinability were given a processing score of 5. Finally, the bread score was a measure for the general outlook of the bread, the crust color, and the crumb characteristics. Coarse crumb structure, lack of or excessive break-and-shred, and bad shape were penalized with a low bread score of 1. Fine, homogeneous crumb, good break-and-shred, and optimal shape were awarded a high bread score of 5. Bread scores complement the loaf volume values.

### Initial Bread Crumb Firmness and Bread Staling

Breadcrumb firmness was determined 24, 48, and 72 hr after baking with a texture analyzer (TA-XT2i, StableMicroSystems, Surrey, UK) according to Approved Method 74-09 (AACC 2000). Three adjacent bread slices of 25 mm were cut from the middle of the bread and put in the middle of the base plate of the texture

TABLE I  
Effects of *Bacillus subtilis* Endoxylanase (XBS) and *Aspergillus aculeatus* Endoxylanase (XAA) Enzyme Dosages (nKat/g of flour) for Flour-A and Flour-B<sup>a</sup>

Enzyme Dosage	BA	BU	Dough Score	Processing Score	Bread Score	Bread Weight	Moisture Content
Flour-A							
XBS							
0.00	59.0	430	4	4	3	835	37.1
0.06	58.5	440	4	4	3	835	37.1
0.13	58.5	480	3	5	4	835	36.9
0.26	58.0	480	3	5	4	830	36.4
0.52	56.0	550	3	5	4	836	36.1
1.04	54.0	580	3	4	4	836	35.3
2.08	53.0	620	3	4	4	832	34.6
XAA							
0.00	59.0	430	4	4	3	835	37.1
0.09	58.5	460	4	4	3	840	37.5
0.23	58.0	440	4	4	3	836	36.8
0.51	58.0	450	3	3	4	831	36.5
1.13	57.5	440	3	3	4	836	36.7
2.63	57.0	420	3	2	3	831	36.1
6.00	55.5	400	3	2	3	837	36.0
Flour-B							
XBS							
0.00	58.0	nd <sup>b</sup>	4	4	3	789	37.9
0.06	58.0	nd	4	5	3	775	36.8
0.13	57.5	nd	3	5	4	783	37.4
0.26	57.5	nd	3	5	4	776	36.8
0.52	57.0	nd	3	4	5	780	37.1
1.04	55.0	nd	4	4	5	769	36.3
2.08	54.0	nd	4	4	4	766	36.0
XAA							
0.00	58.0	nd	4	4	3	789	37.9
0.09	58.0	nd	4	5	3	785	37.6
0.23	57.5	nd	4	4	4	784	37.5
0.51	57.5	nd	4	4	4	784	37.5
1.13	57.0	nd	3	3	3	776	36.8
2.63	56.5	nd	3	2	3	764	35.9
6.00	56.0	nd	3	2	3	764	35.9

<sup>a</sup> Baking absorption (BA, %); farinograph dough consistency (BU); bread weights (g); and bread moisture content (%). Dough, processing, and bread scores range from 1 to 5, with a higher score representing better characteristics.

<sup>b</sup> Not determined.

analyzer. The probe ( $\varnothing = 36$  mm) compressed the crumb 10 mm at a speed of 2 mm/sec. The resistance to further compression when slice thickness was reduced to 18.75 mm was a measure of crumb firmness. The evolution of crumb firmness with time made it possible to quantify staling. Measurements were performed in duplicate, which resulted in six bread slices being measured for each data point (CV < 8%).

### Extraction of AX from Flour, Dough, and Bread Samples

Lyophilized dough and bread samples (5.0 g) were accurately weighed in a centrifuge tube. Deionized water (100 mL, 4°C) was added, and the centrifuge tubes were shaken (30 min, 4°C). After centrifugation (10,000 × g, 15 min, 4°C), the supernatant was transferred into a flask and immediately frozen in liquid nitrogen. The residue in the centrifuge tube was washed with water (50 mL, 4°C) and centrifuged again as above. The supernatant was added to the flask and frozen. After lyophilization, boiling water (150 mL) was poured into the flask, which was then kept in a boiling water bath (30 min) to inactivate the enzyme. The flask was frozen and its contents were lyophilized again. The lyophilized material was dispersed in water (50 mL), centrifuged (10 000 × g, 15 min, 4°C), and frozen until further analysis.

Because of the potentially critical nature of this extraction step as part of the experimental setup, a control procedure was performed in which the enzymes in the samples were inactivated by refluxing in 95% ethanol 2 hr before extraction as above. The results obtained with samples treated this way were virtually identical to those obtained with the nonpretreated samples (results not shown).

### Monosaccharide Content and Composition After Hydrolysis

Monosaccharide composition after hydrolysis of the AX-containing supernatants was estimated by gas liquid chromatography as described by Loosveld et al (1997). Hydrolysis was performed with 2.0M trifluoroacetic acid, reduction with sodium borohydride, and acetylation with acetic anhydride. AX content was then defined as 0.88× the sum of the monosaccharides xylose and arabinose (corrected for the presence of arabinogalactan-peptide) to account for uptake of water during hydrolysis. Polymeric glucose content

was defined as 0.9× monosaccharide glucose content. The complete procedure, from breadmaking and extraction to monosaccharide composition had a CV < 4.5%.

### High-Performance Size-Exclusion Chromatography

Supernatants (20 μL) were separated on a Shodex SB-806 HQ high-performance size-exclusion chromatography (HPSEC) column (300 × 8 mm i.d.) with a Shodex SB-G guard column (50 × 6 mm i.d.) from Showa Denko K.K. (Tokyo). Elution was with 0.3% NaCl (0.5 mL/min at 30°C) on a pump system (325, Kontron, Milan, Italy) with autoinjection. The separation was monitored with a refractive index detector (VSD Optilab, Berlin, Germany). Molecular weight markers were Shodex standard P-82 pullulans (1.0 mg/mL) with molecular weight of 78.8 × 10<sup>4</sup>, 40.4 × 10<sup>4</sup>, 21.2 × 10<sup>4</sup>, 11.2 × 10<sup>4</sup>, 4.73 × 10<sup>4</sup>, 2.28 × 10<sup>4</sup>, 1.18 × 10<sup>4</sup>, and 0.59 × 10<sup>4</sup> as well as glucose.

To determine the change in peak molecular weight of AX in solution compared with the control, the HPSEC-profile of the control was subtracted from each of the profiles obtained for enzyme-supplemented samples. The approach yields a differential chromatogram, from which peak molecular weight estimates can be read easily. Because the addition of endoxylanases does not alter the non-arabinose and nonxylose sugar composition of the aqueous extracts, this method of processing the HPSEC profiles is justified.

### Viscosity Measurements

Viscosity of the extracts was determined with an Ostwald-type viscosimeter at 30°C according to Vinkx et al (1991). Average triplicate viscosity measurements had CV 1.1%, with a maximum of 3.1%.

### SDS-Unextractable Protein in Dough and Bread Samples

Extraction of lyophilized dough and bread samples was performed as described by Veraverbeke et al (1999). Samples (1.5 g)

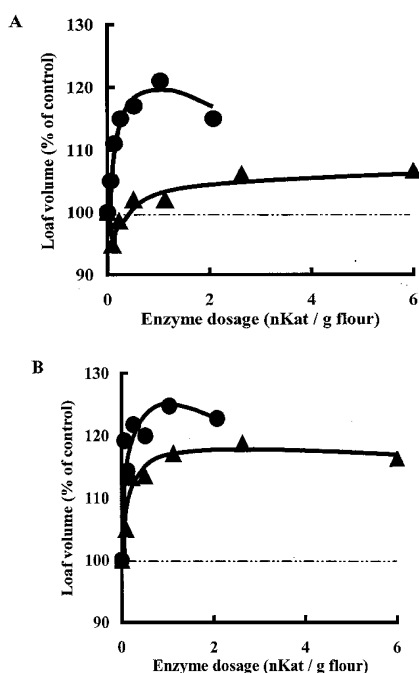


Fig. 1. Specific loaf volume for bread from flour-A (A) and flour-B (B) with added *Bacillus subtilis* endoxylanase (●) and *Aspergillus aculeatus* endoxylanase (▲). Control bread loaf volumes were 3,050 and 3,330 cm<sup>3</sup>, respectively.

TABLE II

Molecular Weight of AX<sup>a</sup> in Flours After Addition of *Bacillus subtilis* Endoxylanase (XBS) and *Aspergillus aculeatus* Endoxylanase (XAA)

Enzyme Dosage <sup>b</sup>	After Mixing	After Fermentation	After Baking
Flour-A			
XBS			
0.06	190,000	145,000	67,000
0.13	163,000	58,000	64,000
0.26	76,000	53,000	49,000
0.52	47,000	35,000	37,000
1.04	27,000	19,000	27,000
2.08	24,000	9,000	18,000
XAA			
0.09	35,000	41,000	37,000
0.23	31,000	15,000	35,000
0.51	31,000	12,000	19,000
1.13	13,000	6,000	10,000
2.63	6,000	3,000	3,000
6.00	...	2,000	2,000
Flour-B			
XBS			
0.06	202,000	64,000	41,000
0.13	154,000	58,000	41,000
0.26	56,000	41,000	37,000
0.52	56,000	38,000	39,000
1.04	38,000	13,000	31,000
2.08	21,000	7,000	12,000
XAA			
0.09	37,000	15,000	26,000
0.23	45,000	17,000	24,000
0.51	35,000	12,000	20,000
1.13	24,000	7,000	10,000
2.63	5,000	4,000	6,000
6.00	4,000	3,000	4,000

<sup>a</sup> High-performance size-exclusion chromatography peak molecular weight data of arabinoxylan in dough and bread aqueous extracts.

<sup>b</sup> nKat/g of flour.

were suspended in 1.5% (w/v) SDS solution (30 mL). The suspension was shaken gently for 2 hr to avoid excessive foaming. An aliquot (4.0 mL) of the supernatant obtained after centrifugation (30,000 × g, 30 min) was mixed with sulfuric acid (0.5 mL) and concentrated by heating for 1 hr at 150°C. Protein content (N × 5.7) was determined by a semimicro-Kjeldahl method. The amount of SDS-unextractable protein was calculated by difference. Average triplicate Kjeldahl nitrogen determinations had CV 1.3%, with a maximum of 1.5%.

## RESULTS

### Dough and Bread Characteristics

**Dough.** Dough and bread properties and processing conditions for XBS and XAA in both breadmaking systems are summarized in Table I. Dough stickiness was the main factor causing the manually determined baking absorption values to decrease with increasing enzyme concentrations. It occurred especially at high enzyme levels, and more so for XBS than for XAA. The farinograph consistency measurements for the XBS and flour-A combination shows that the farinograph is not a good tool for predicting baking absorption at higher XBS dosages. Correction of the baking absorption to obtain equal farinograph consistencies for all doughs would have led to increasing stickiness with increasing enzyme concentrations. For XAA, the dough consistency measured with the farinograph agreed much better with the evaluation by the experienced baker. Overall, dough scores did not change markedly. A small decrease was observed for XBS and was caused mainly by an increase in dough stickiness. For XAA, dough scores were equal to or somewhat better than the control. For dough processing, the opposite trend was observed. Manageability increased somewhat with XBS addition, while it decreased for XAA. The latter was especially clear at the two highest XAA enzyme dosages.

**Bread.** Both enzymes, at most of the enzyme concentrations used, led to an improved overall bread score. This was mainly because of better crumb structure and general bread characteristics, such as break-and-shred and loaf shape.

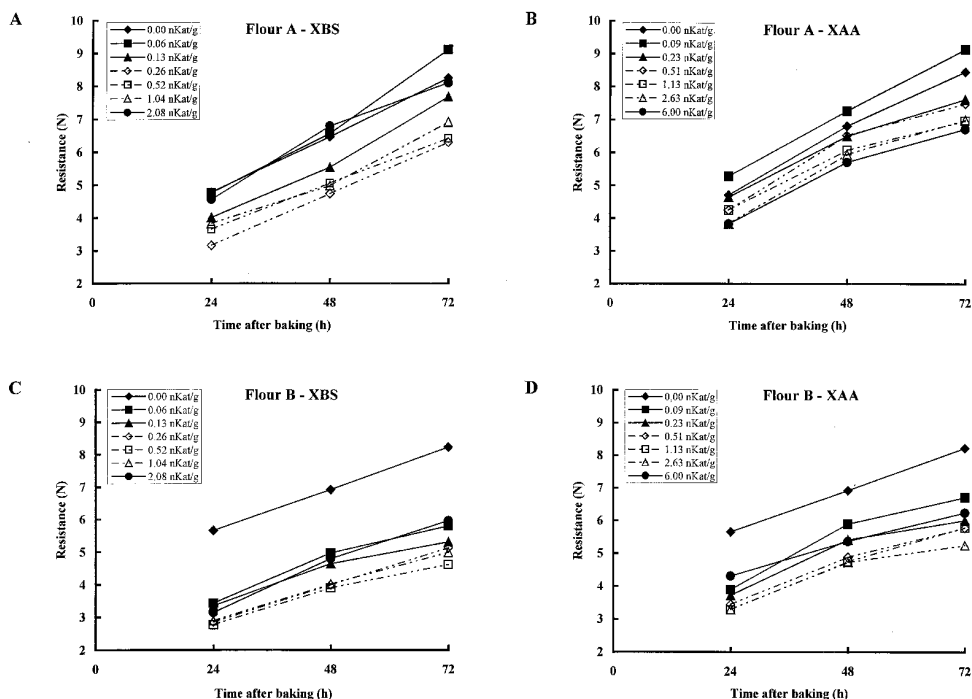
Specific loaf volumes (cm<sup>3</sup>/g of bread) are given in Fig. 1A and B. For XBS, a gradual increase in specific loaf volume was evident in the flour-A breadmaking process (Fig. 1A). Only at the highest dosage (2.08 nKat/g of flour) was a setback observed. This was probably due to the low baking absorption, resulting in a somewhat impeded gluten development. For XBS in the flour-B breadmaking process (Fig. 1B), maximum loaf volume was reached almost immediately.

XAA showed different effects. For flour-A (Fig. 1A), specific loaf volume decreased upon addition of XAA at the lowest dosage (0.09 nKat/g flour). This decrease, although small, was statistically significant. Only at the two highest enzyme concentrations (2.63 and 6.00 nKat/g flour) were small increases in specific loaf volume observed. With flour-B (Fig. 1B), a specific loaf volume increase of 6–19% was noted. As a general trend, changes in moisture content of the loaves followed the changes in baking absorption (Table I).

**Initial crumb hardness and staling.** Results of bread crumb firmness measurements as a function of storage time are summarized in Fig. 2A–D. Initial crumb firmness apparently correlates rather well with loaf volume. For XBS addition to flour-A (Fig. 2A), a decrease in firmness was observed for dosages from 0.13 to 1.04 nKat/g of flour. The lowest dosage (0.06 nKat/g of flour) had little effect on loaf volume and, equally, had little effect on crumb firmness. For the highest dosage (2.08 nKat/g of flour), resistance equal to that of the control can probably be ascribed to both loaf volume and low moisture content of the bread loaves. Crumb firmness increased for the lowest dosage (0.09 nKat/g of flour) of XAA added to the same flour (Fig. 2B). This agreed with the observation that loaf volume is lower than that of the control. For the other loaves, initial crumb firmness decreased steadily with increasing enzyme addition.

Except for the large difference between the crumb firmness for the control and the enzyme-containing breads, the results for XBS and XAA with flour-B (Fig. 2C and D) were similar to those for flour-A.

It is not clear how to explain lower crumb firmness with both XBS and XAA. In part, the phenomena are due to the effect of the enzymes on loaf volume. However, although the changes in crumb



**Fig. 2.** Resistance to compression of bread crumbs vs. time after baking for flour-A breads with various doses of *Bacillus subtilis* endoxylanase (XBS) (A) and *Aspergillus aculeatus* endoxylanase (XAA) (B); and for flour-B with XBS (C) and for flour-B with XAA (D).

firmness went hand-in-hand with the changes in loaf volume, it is not clear whether only the variation of the latter can cause the large differences in crumb firmness. Indeed, for flour-B breads, the difference in crumb firmness between the control and breads with optimal enzyme concentration was almost twofold, whereas the volume difference was only  $\approx 20\%$ . The decrease in moisture content of the loaves with increasing enzyme dosages on virtually all occasions clearly cannot explain the remainder of the difference (Table I). Additional factors involved may be thickness of the crumb cell walls and size distribution of crumb pores. WU-AX is a structural component of cereal grains contributing to strength; therefore, the enzymic breakdown of this material might be a factor involved as well.

The evolution of bread crumb firmness with storage time, which is an indication for the rate of staling, was similar for all loaves made with both enzymes in both processes. This would suggest that the staling rate is not influenced by the addition of XBS or XAA, which is in contrast to the findings of Martinez-Anaya and Gimenez (1997).

### Properties of AX in Dough and Bread

**Solubilization.** Adding endoxylanase to breadmaking recipes solubilizes WU-AX during the breadmaking process. The effect depends on the concentration of enzyme added and the selectivity of the enzyme. Figure 3A-B shows the solubilization of WU-AX in flour-A doughs and breads with XBS and XAA, respectively. The first and most obvious difference is that XBS solubilized most of the WU-AX during mixing and at low enzyme concentrations, whereas solubilization with XAA occurs mainly during fermentation and at higher enzyme dosages.

A second difference lies in the curves representing solubilized AX after baking. For XBS, the curve is positioned under the curves for mixed and fermented doughs. This indicates that, during baking, part of the previously solubilized AX was turned unextractable again. This is probably due to chemical crosslinking of AX molecules with themselves or other flour components, or physical inclusion. Whatever the case, there is evidence for chemical crosslinking in the HPSEC profiles. For XAA, the level of ES-AX after baking was higher than after mixing and higher or equal to the level after fermentation. This implies that solubilization by XAA progresses

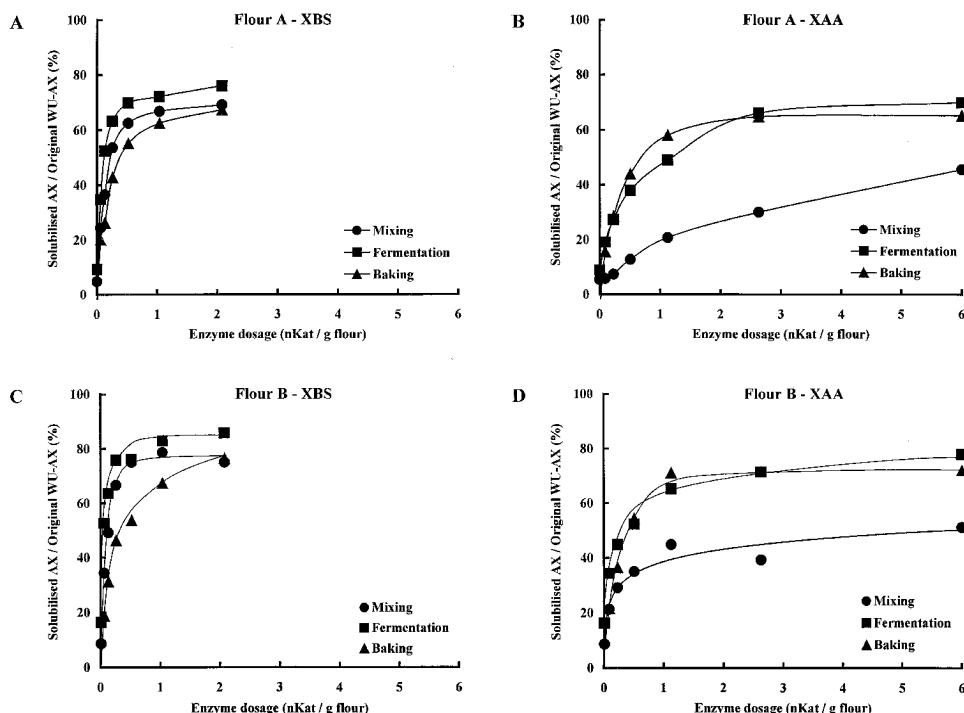
during baking and that the AX fragments formed by XAA are not retained in the bread structure during extraction. The most probable explanation for the latter is that the fragments are smaller than those formed by XBS. It seems reasonable that short fragments are rendered less unextractable by covalent crosslinking and that they would be less easily trapped in the gluten-starch matrix.

A third but smaller difference is the extent to which the WU-AX was solubilized. At the highest dosage, XBS solubilized  $\approx 80\%$  of the WU-AX after fermentation, whereas, XAA solubilization seemed to level off at 70%. On the one hand, both values are substantially higher than what was observed by Rouau et al (1994), who found a solubilization level at  $<40\%$ . This discrepancy is easily explained by lower enzyme dosages. On the other hand, their results agree rather well with the observations by Rouau and Moreau (1993) and Gruppen et al (1993) that maximally 75% of WU-AX can be enzymically solubilized in vitro.

Solubilization trends during flour-B processing (Fig. 3C and D) are very similar to those observed for flour-A. A higher degree of solubilization was evident in flour-B. A faster solubilization during mixing in XAA also was observed. However, the extent to which the use of faster spiral mixing instead of slower arthofex mixing, the different flours, and the different fermentation times are responsible for both observations cannot be deduced from the available data.

**HPSEC analysis and molecular weight of AX.** The solubilization results are complemented with HPSEC molecular weight profiles of AX in the aqueous extracts (Fig. 4). In addition, Table II shows apparent peak molecular weight data obtained by subtracting the HPSEC profiles of the control from the HPSEC profiles of the enzyme-supplemented samples.

Analysis of the data for flour-A shows that, during mixing, XBS solubilized fragments with a molecular weight of 25,000 to 200,000 at low and intermediate dosages (Fig. 4A). There was no decrease in refractive index response measured in the high molecular weight region ( $\geq 400,000$ ), which indicates that no WE-AX was degraded under these conditions. At the highest enzyme levels, molecular weight fragments of  $\approx 20,000$  were formed. After fermentation, a general shift toward lower molecular weights was observed (Fig. 4B).



**Fig. 3.** Solubilized water-unextractable arabinoxylan (WU-AX) after mixing (●), fermentation (■), and baking (▲) for flour-A with various doses of *Bacillus subtilis* endoxylanase (XBS) (A) and *Aspergillus aculeatus* endoxylanase (XAA) (B); and for flour-B with XBS (C) and for flour-B with XAA (D).

During baking, molecular weight rose again, suggesting the formation of diferulic acid or other bridges between separate AX molecules (Fig. 4C) or linking of AX molecules to other biopolymers. In low XAA dosages, degradation of native WE-AX with a high molecular weight was mainly responsible for the AX fragments with molecular weights of  $\leq 50,000$  after mixing because solubilization was low to nonexistent (Fig. 4D). At higher XAA dosages, fragments of 3,000–4,000 were obtained. Similar profiles were found after fermentation (Fig. 4E). Also with XAA, molecular weights rose after baking compared with fermentation, again suggesting the formation of bridges between AX fragments (Fig. 4F).

The profiles for samples made from flour-B showed trends similar to those for flour-A (Table II, profiles not shown). In general, degradation seemed to have progressed somewhat further in the former than in the latter. This can, at least partially, be ascribed to the longer fermentation times used in the flour-B breadmaking process. The use of a spiral mixer instead of an arthoflex mixer also may have caused better interaction between enzyme and substrate during mixing due to its stronger mixing action.

**Viscosity.** Specific viscosity determinations provide an indication of the actual effect of both AX concentration and molecular weight on the viscosity of the dough liquor phase. While an increase in ES-AX concentration increases specific viscosity, degradation of WE-AX and ES-AX will decrease it. Comparison of the data in Fig. 5A and C for flour-A and flour-B, respectively, shows that addition of XBS to the breadmaking process led to an increased specific viscosity at low enzyme dosages. After reaching a peak, viscosity decreased to values below the original viscosity. A similar observation was made by Petit-Benvegnen et al (1998). For XAA (Fig. 5B and D for flour-A and flour-B, respectively), the drop in viscosity was immediate. No increase was observed here. Both phenomena are easily explained on the basis of the results by Courtin and Delcour (2001) and the solubilization and molecular weight data above. For XBS, solubilization of large AX fragments is prevalent over degradation of WE-AX or ES-AX at low enzyme dosages, resulting in an increase in extract viscosity. At higher enzyme levels, where most of the solubilization has already occurred, degradation is the main event taking place. Viscosity then drops again. In con-

trast, XAA preferentially degrades WE-AX in the dough, causing viscosity to drop immediately. During WU-AX degradation, fragments with low molecular weight are released and have little or no impact on viscosity. Rouau et al (1994), who added an endoxylanase-containing enzyme mixture to bread, also observed a rise in specific viscosity upon addition of the mixture. They probably did not note a decrease in specific viscosity because of the relatively low enzyme concentrations they used. Indeed, their maximal dosage solubilized <40% of the WU-AX at the end of fermentation.

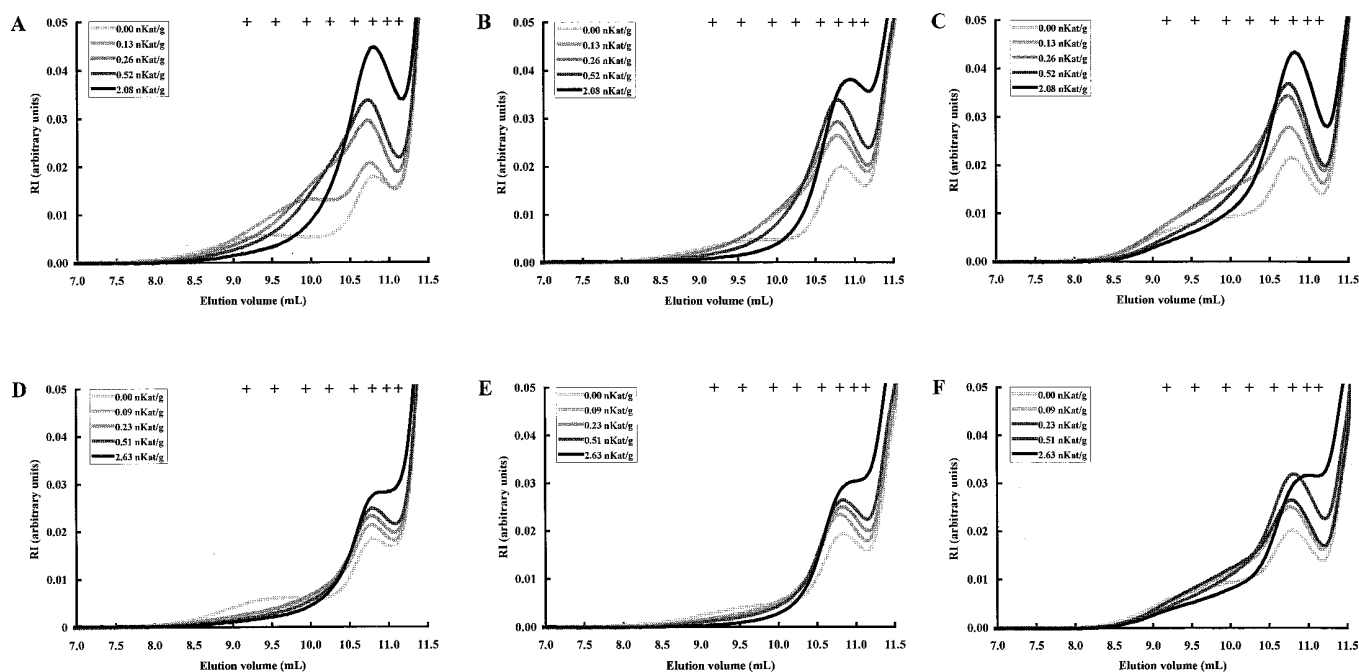
### SDS-Unextractable Protein

On several occasions, it was hypothesized that AX functionality in breadmaking, be it negative (Roels et al 1993) or positive (Sprössler 1997), is the result of interactions of AX with wheat flour gluten proteins. These are important components in wheat flour breadmaking because they are responsible for the dough's viscoelastic network. In addition, the level of the most strongly aggregating, largest glutenin polymers was a good measure of breadmaking potential (Orth and Bushuk 1972; Gupta et al 1993). Such proteins are unextractable in SDS solutions. Therefore, as in the work of Veraverbeke et al (1999), measuring the amount of SDS-unextractable protein was selected as a method for evaluating changes in gluten aggregation due to endoxylanase action.

Levels of SDS-unextractable protein in flour-A dough and bread samples were similar for control and enzyme-supplemented doughs and breads, irrespective of the dosage and the enzyme added. After mixing, 17% of the dough protein content was unextractable. This rose to 25% after fermentation and 89% after baking. The latter is caused by the extreme denaturing conditions of the baking process. These results would suggest that the endoxylanases have no effect on the formation or aggregation of large glutenin molecules. However, the straightforward experimental setup does not rule out other interactions.

## DISCUSSION

Breadmaking with endoxylanases of different substrate selectivity and characterizing the AX population at different steps in the



**Fig. 4.** High-performance size-exclusion chromatography molecular weight profiles of the arabinoxylan in aqueous extracts. **A**, Dough; **B**, fermented dough; and **C**, bread made from flour-A with increasing *Bacillus subtilis* endoxylanase dosages. **D**, Dough, **E**, fermented dough, and **F**, bread made from flour-A with increasing *Aspergillus aculeatus* endoxylanase dosages. Elution volumes of pullulan standards of molecular weights  $78.8 \times 10^4$ ;  $40.4 \times 10^4$ ;  $21.2 \times 10^4$ ;  $11.2 \times 10^4$ ;  $4.73 \times 10^4$ ;  $2.28 \times 10^4$ ;  $1.18 \times 10^4$ , and  $0.59 \times 10^4$  are indicated from left to right.

process were intended to evaluate whether our previous observations on AX and endoxylanase functionality in breadmaking (Courtin and Delcour 1998, 2001; Courtin et al 1999) would also apply to industrial breadmaking and whether AX modifications have an impact on bread characteristics other than loaf volume. We predicted that endoxylanases with different relative activity toward WE-AX and WU-AX would behave differently in breadmaking (Courtin et al 1999). The results from these experiments corroborate previous findings.

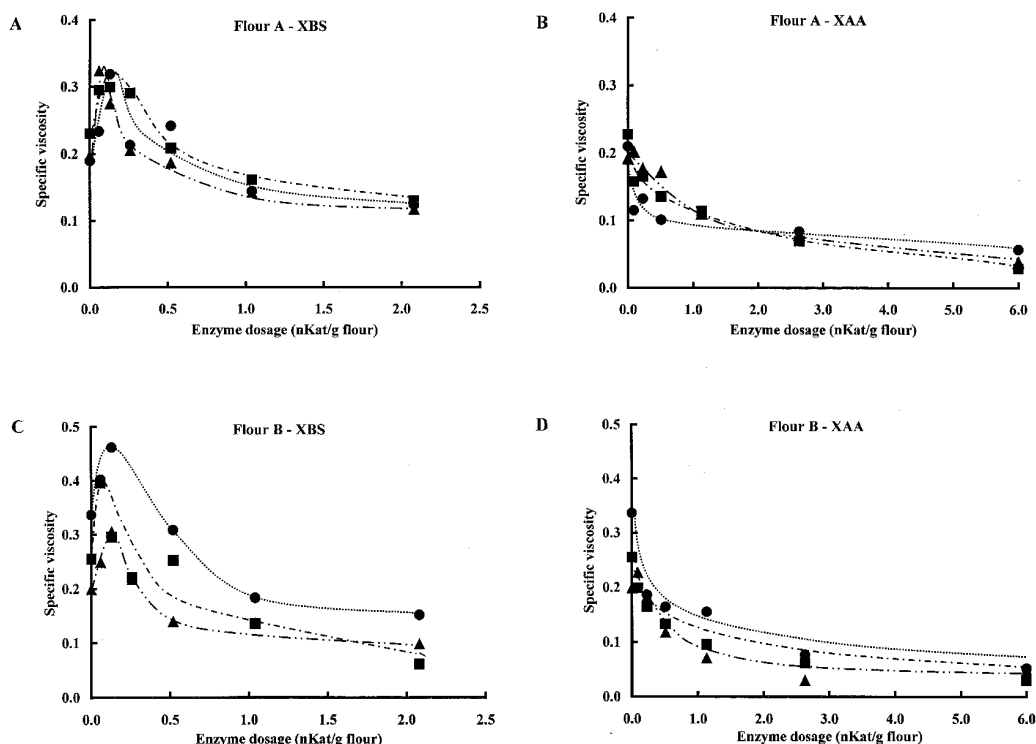
At low enzyme concentrations (0.06–0.25 nKat/g of flour), relevant for industrial breadmaking, XBS rapidly released AX fragments of intermediate molecular weight (50,000–200,000) from the WU-AX, whereas the WE-AX was left seemingly unaffected. This resulted in an increased viscosity in the dough liquor phase, which partially compensated for the loss in consistency due to the lower total water holding capacity of the WU-AX population, which was evident from the small decrease in baking absorption at low enzyme concentrations. During fermentation, additional solubilization was accompanied by some further degradation of the WE-AX and ES-AX. These effects were more noticeable for the breadmaking process with flour-B, in which fermentation time was almost twice as long as for flour-A. In the oven, lowering AX molecular weight by further enzymic degradation was probably masked by the formation of longer molecules through crosslinking of AX fragments. At this stage, part of the WE-AX and ES-AX was trapped in the starch-gluten structure by chemical or physical interactions. The loaf volume increases were 5–20% for flour-A and ≈20–25% for flour-B.

When high levels of XBS were added (0.52–2.08 nKat/g of flour), solubilization during mixing was very high and started leveling off at the highest dosage, while degradation of WE-AX and ES-AX continued to peak molecular weights of 10,000–50,000. This resulted in a considerable decrease in baking absorption and a decrease of extract viscosity compared with the control. The fact that, under these conditions, further increases in loaf volume and improvements of crumb and bread characteristics were observed strongly indicates that endoxylanase functionality is not only governed by the production of ES-AX but also, and perhaps mainly, by the removal of WU-AX. In addition, timing might be an important factor.

At low XAA concentrations (0.09–0.51 nKat/g of flour), solubilization during mixing was very low for flour-A, while it was more pronounced in doughs made from flour-B. In both cases, lower viscosity of dough extracts was observed. Molecular weight profiles showed that this was caused by degradation of the WE-AX. The relatively low level of low molecular weight ES-AX fragments did not counteract this effect. Baking absorption was slightly depressed. During fermentation, conversion of WU-AX to solubilized AX increased strongly. ES-AX fragments with relatively low molecular weight were formed. The combination of both events increased the level of free water in the dough, making it slacker and more sticky. Hydrolysis continued in the oven. For flour-A, this modification of the AX population resulted in an initial decrease in loaf volume followed by a small increase with increasing enzyme concentration. For flour-B, loaf volume increased 5–19%.

At high XAA concentrations (1.13–6.00 nKat/g of flour), WU-AX solubilization increased further during mixing but never reached the levels obtained with XBS. Baking absorption was depressed somewhat. Considerable solubilization and extensive degradation mainly occurred during fermentation, and the additional water released from the WU-AX resulted in very poor dough processability. In spite of this, loaf volume increase was higher than for the low XAA dosages: 6% for flour-A and 19% for flour-B. ES-AX fragments had low molecular weight, and viscosity of the dough aqueous phase was low. Again, this pointed more to the importance of WU-AX degradation than to ES-AX formation.

The somewhat less pronounced reaction of flour-A to endoxylanase addition compared with flour-B can be tentatively attributed to several factors including mixing intensity, fermentation time, flour composition, and different levels of xylanase inhibitors (Gebruers et al 2001). At low enzyme dosages, higher loaf volumes levels were accompanied by higher solubilization levels, which favors the idea that the difference is process-related. The difference in solubilization after mixing for XAA added to flour-A and flour-B was especially remarkable. On the other hand, the difference in loaf volume between flour-A and flour-B at high XAA dosages might be flour-related because solubilization patterns were quite similar at this



**Fig. 5.** Specific viscosities of the aqueous extracts of dough (●), fermented dough (■), and bread (▲) for flour-A with various doses of *Bacillus subtilis* endoxylanase (XBS) (A) and *Aspergillus aculeatus* endoxylanase (XAA) (B); and for flour-B with XBS (C) and for flour-B with XAA (D).

point. Reaction of flours to endoxylanase treatment is flour-dependent, with weaker flours responding better than stronger ones (Rouau et al 1994).

The impact of AX (WE-AX or WU-AX) on bread staling is a controversial issue and seems to depend strongly on water content in the bread system (Kim and D'Appolonia 1977; Eliasson and Larsson 1993; Biliaderis et al 1995). From the present results, it can be concluded that the modification of the AX population by endoxylanases only alters initial bread crumb firmness, while staling is left unaffected. The changes in firmness seem to be strongly related to loaf volume but less so to water content of the bread loaves. This finding is in contrast with the report by Martinez-Anaya and Jimenez (1997), who stated that staling decreased when endoxylanases were used.

Evaluation of baking absorption for each enzyme addition separately allowed use of low to high dosages. A baking absorption equal to that of the control for all doughs would have prevented the work of high levels of either XBS or XAA, where solubilization is maximal and degradation is significant. Excessive release of water would have resulted in doughs that were too slack and sticky at the mixing stage. In industrial breadmaking, a decrease in baking absorption should be avoided because it decreases dough yield.

## CONCLUSIONS

The results obtained with the present approach largely corroborate previous findings from this group. The endoxylanase with selectivity toward WU-AX (XBS) did indeed perform better than the endoxylanase with selectivity toward WE-AX (XAA). However, under certain conditions, the latter also gave acceptable and improved breads. However, at higher enzyme dosages, processability with XAA was seriously hampered. Taken together with evidence found in literature, our results show that the solubilization of WU-AX to ES-AX is beneficial for breadmaking, not only because of the increase in ES-AX in the dough liquor phase, but also mainly because of the removal of WU-AX. Furthermore, it can be assumed that endoxylanase functionality, and thus most probably also AX functionality, depends in part on processing conditions and flour characteristics.

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