Fractionation−Reconstitution Experiments Provide Insight into the Role of Endoxylanases in Bread-Making

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The impact mechanism of endoxylanases in straight dough bread-making was investigated in fractionation−reconstitution experiments. To this end, two European flours with different bread-making characteristics were separated in gluten, prime starch, a squeegee fraction (SQF), and a water-extractable fraction. Whereas the former fractions contained negligible levels of arabinoxylan (AX), the latter contained, respectively, most of the water-unextractable AX (WU-AX) and all of the water-extractable AX (WE-AX). In vitro modification with a *Bacillus subtilis* endoxylanase allowed controlled solubilization of WU-AX from SQF and controlled degradation of solubilized AX and WE-AX from the water-extractables. It followed from bread-making tests with the reconstituted flours that endoxylanases exert positive loaf volume effects in bread-making by lowering the concentration of WU-AX and increasing that of total soluble AX. Limited degradation of WE-AX and significant breakdown of solubilized AX by endoxylanases, on the other hand, resulted in volume losses when compared to their nondegraded counterparts. The volume increasing effects of endoxylanases are therefore related to their ratio of solubilizing to degrading activity and thus to their substrate specificity.

Keywords: *Wheat; endoxylanase; arabinoxylan; bread-making*

INTRODUCTION

Most of the research on AX functionality indicates that AX plays a prominent role in bread-making (Cawley, 1964; Casier et al., 1973; McCleary et al., 1986; Kühn and Grosch, 1989; Michniewics et al., 1992; Vanhamel et al., 1993; Rouau et al., 1994; Biliaderis et al., 1995). However, the functionality of AX is, in general, far from being well understood. This is caused, on the one hand, by the coexistence of water-extractable (WE-AX) and water-unextractable AX (WU-AX) and, on the other hand, by the exceptional combination of physicochemical properties of the AX, of which variability in structure both within WE-AX and within WU-AX, high water-holding capacity, and gelling characteristics are well-known. An additional confusing factor is that the term WU-AX is often used for both native WU-AX and isolated WU-AX that, during the isolation process, in many instances, becomes water-soluble (Casier et al., 1973; Krishnaray an Hoseney, 1994). In this paper we use the term WU-AX when considering AX in its original insoluble form and solubilized AX when dealing with WU-AX solubilized by either alkaline treatment or enzymic hydrolysis.

One of the tools used in understanding AX functionality is AX-degrading enzymes and, more specifically, endoxylanases. However, the use of such enzymes in bread-making recipes has, to date, complicated the original question. McCleary et al. (1986) reported that the use of an endoxylanase in bread-making resulted in sticky doughs and loss of good crumb characteristics and loaf structure. Loaf volume increased because of large air bubbles in the crumb. In contrast, Rouau et al. (1994) and Krishnaray and Hoseney (1994) showed that addition of endoxylanases can indeed increase bread volume, without detrimental effects on bread and crumb structures. The reason for these differences is not clear. Neither is the mechanism by which endoxylanases influence bread-making. Rouau et al. (1994) suggested that an increased soluble AX level was probably the reason for increased bread quality upon addition of an endoxylanase-containing preparation.

It is evident that the influence of endoxylanases in bread-making is to be ascribed to their role in the modification of the dough AX population. From a theoretical viewpoint, they can do so in five distinct ways. First of all, endoxylanases can cut WU-AX xylan backbones without solubilizing them (Gruppen et al., 1993). Second, they can reduce the level of WU-AX present by solubilizing them. This, in a third step, leads to an increase in the level of solubilized AX. Fourth, endoxylanases reduce the molecular weight of the solubilized AX and, fifth, also of the WE-AX.

It is further obvious that, in bread-making, some if not all of these five components of endoxylanase activity occur simultaneously and to variable extents. The relative rate at which these processes occur depends on different factors such as the enzyme specificity and the physicochemical characteristics of the AX population.

In this paper, the well-established technique of fractionation−reconstitution of flour, which, in the past, has proven to be very useful for our understanding of the role of specific flour components in bread-making (Finney, 1943; Hoseney et al., 1969; MacRitchie, 1985), is combined with controlled in vitro modification of AX in different flour fractions. All but one of the aforementioned endoxylanase activities were tested. The enzymic

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Table 1. Moisture (Percent), Yields (Expressed as Percent of the Sum of Yields, dm), Protein (Percent dm) and Ash Contents (Percent dm), Monosaccharide Compositions (Percent dm), AX and Starch Contents (Percent dm), and A/X of the Fractions Obtained after Fractionation of Soissons and Torfrida Flours

fraction	moisture	yield	protein	ash	Ara	Xyl	Man	Gal	Glc	A/X[b]	AX[e]	WU-AX[f]	starch[g]
(a) Soissons													
native flour	13.60	100	10.3	0.50	0.74	1.02	0.23	0.37	82.98	0.58	1.42	100	74.68
prime starch	7.80	61.52	nd[a]	0.16	0.09	0.14	0.09	0.04	102.02	0.53	0.20	11.1	91.82
SQF	7.90	19.97	1.33	0.21	2.01	3.27	0.31	0.13	91.13	0.59	4.56	82.0	82.02
gluten	6.33	13.65	77.39	0.98	0.42	0.41	0.14	1.45	14.72	nd[c]	0.56	6.9	13.24
WEF	10.47	4.86	21.43	5.07	5.18	5.26	2.80	3.96	35.87	0.54[d]	7.13	0.0	32.28
(b) Torfrida													
native flour	12.79	100	11.1	0.67	1.01	1.63	0.24	0.44	81.17	0.53	2.15	100	73.05
prime starch	3.19	56.26	nd[a]	0.43	0.11	0.16	0.08	0.03	99.70	0.56	0.23	7.8	89.73
SQF	7.14	23.60	1.26	0.35	2.57	4.55	0.31	0.11	91.33	0.56	6.19	87.6	82.20
gluten	3.99	13.18	80.53	0.92	0.43	0.45	0.14	1.14	11.10	nd[c]	0.58	4.6	9.99
WEF	10.86	6.96	19.56	4.16	4.17	5.43	4.18	2.88	52.78	0.47[d]	7.02	0.0	42.34

[a] nd, not determined. Protein content of the prime starch fractions was to low to be accurately measured by the technique used. [b] A/X = [(Ara − (Gal × 0.7))/Xyl]. Due to galactolipids, the WE fraction galactose content is used instead of the flour galactose content for calculating A/X in flour. [c] Due to galactolipid presence, gluten A/X could not be calculated. [d] A/X ratios determined for isolated WE-AX. [e] AX = [Xyl + (Xyl × A/X)] × 0.88. [f] WU-AX, yield of WU-AX expressed as percentage of the total amount of WU-AX present in flour. [g] Starch = glucose × 0.9.

breakdown of WU-AX without solubilization could indeed not be accurately simulated with the experimental approach used.

It was our firm belief that, by recombining original flour fractions with enzyme-treated ones and with isolated WE-AX, the effect of each of these components could be assessed individually and that this would increase our understanding of the functionality of AX as a whole and of endoxylanases in particular.

We here report on the outcome of this work.

MATERIALS AND METHODS

Chemicals. *Bacillus subtilis* endoxylanase, free from amylase, glucanase, and protease activities, was obtained from Puratos NV (Groot-Bijgaarden, Belgium). One unit of this enzyme preparation is defined as the amount of enzyme needed (microliters) to give a change in extinction of 1.0 at pH 6.0 in the azurine cross-linked arabinoxylan procedure described in Megazyme Data Sheet 8/94 (Megazyme, Bray, Ireland). All reagents were of at least analytical grade and supplied by Sigma-Aldrich (Bornem, Belgium) unless specified otherwise. Specialty chemicals were heat-stable α-amylase solution (Thermamyl LS 120, Novo Nordisk, Bagsvaerd, Denmark) and amyloglucosidase (Boehringer Mannheim, Mannheim, Germany). Standard P-82 pullullans were purchased from Showa Denko K.K. (Tokyo, Japan).

Analytical Methods. Monosaccharide compositions were estimated by gas−liquid chromatography of alditol acetates following hydrolysis, reduction, and derivatization as described by Loosveld et al. (1997) with, for calculation of AX present, correction for the presence of arabinogalactan peptides (AGP) based on an arabinose-to-galactose ratio of 0.7. AX content was defined as 0.88 times the sum of monosaccharide xylose and corrected arabinose. The arabinose-to-xylose ratio (A/X) was also calculated with the corrected arabinose content (Loosveld et al., 1997; see also Table 1). A micro-Kjeldahl procedure [AACC Method 46.13 (N × 5.7)] was used for protein assessment. Moisture and ash contents were determined according to AACC Methods 44.15A and 08.01, respectively. Farinograph water absorption was measured according to AACC Method 54.21 and was based on dough consistency at the 500 Brabender unit line.

Flours. Wheats (Soissons and Torfrida, harvest 1996) were obtained from AVEVE (Landen, Belgium). Conditioning was at 14.5% moisture. The samples were milled on a Buhler MLU-202 laboratory mill (Uzwill, Switzerland) according to AACC Method 26.31. Milling yields, protein contents (percent dm), ash contents (percent dm), and farinograph water absorption (14% moisture base) were 69.4, 74.2, 10.3, and 11.1% and 0.50, 0.67, 55.8, and 60.7% for Soissons and Torfrida flours, respectively.

Fractionation of Wheat Flour. Fractionation was based on the procedure of MacRitchie (1985) and carried out five consecutive times with 250 g quantities of both Soissons and Torfrida flours. Each 250 g quantity was kneaded manually to doughs of comparable consistency, with 150 and 160 mL of deionized water, respectively. Gluten was recovered by five subsequent washings with 500 mL of deionized water (15 °C). Starch and the water-extractable fraction were separated by centrifugation of the washing liquor (5000*g*, 10 min, 15 °C). The starch was then divided into prime starch and the squeegee fraction (also known as squeegee starch, starch tailings, or B-starch and further referred to as SQF) by carefully scraping off the darker SQF layer from the white prime starch in the centrifuge tubes. The SQF was again suspended in 250 mL of deionized water and centrifuged as above to remove residual prime starch. Both were separated again, and the supernatant was added to the water-extractables. The four fractions were lyophilized and homogenized with a mortar and pestle, with special attention not to damage the starch granules. All fractions were sieved through a 250 μm sieve.

The four different fractions resulting from five fractionation experiments were combined to obtain a homogeneous fractionation lot. They will further be referred to as prime starch, gluten, SQF and water extractable fraction (WEF).

Enzymic Modification of WU-AX in the SQFs. *Preliminary Experiments.* To assess the optimal incubation time needed for solubilizing a maximal amount of WU-AX with minimal breakdown of the polymeric structure of the AX, five aliquots of Torfrida and Soissons SQF (each 1.50 g) were suspended in 10.0 mL of NaCl solution (0.3% w/v) containing 0.06 unit of *B. subtilis* endoxylanase. The mixtures were incubated at 30 °C under continuous shaking. After 10, 30, 90, 240, and 480 min incubations, respectively, they were centrifuged (9000*g*, 10 min, 15 °C). The hydrolysis of the five mixtures was ended by placing the supernatant in boiling water for 10 min, which reduced the enzyme activity (Megazyme azurine cross-linked arabinoxylan procedure) to <5% of its original value. The residue was washed with water, centrifuged as above, suspended in water, and lyophilized. The obtained supernatants were combined. After a second centrifugation step as above, the supernatant was analyzed by gel permeation chromatography (cfr. infra) and gas chromatography as outlined above. A control sample, which lacked the enzyme, was incubated for 30 min at 30 °C and received the same treatment as described above.

Large-Scale Treatment. The results of the above experiments led to the selection of conditions for larger scale incubations

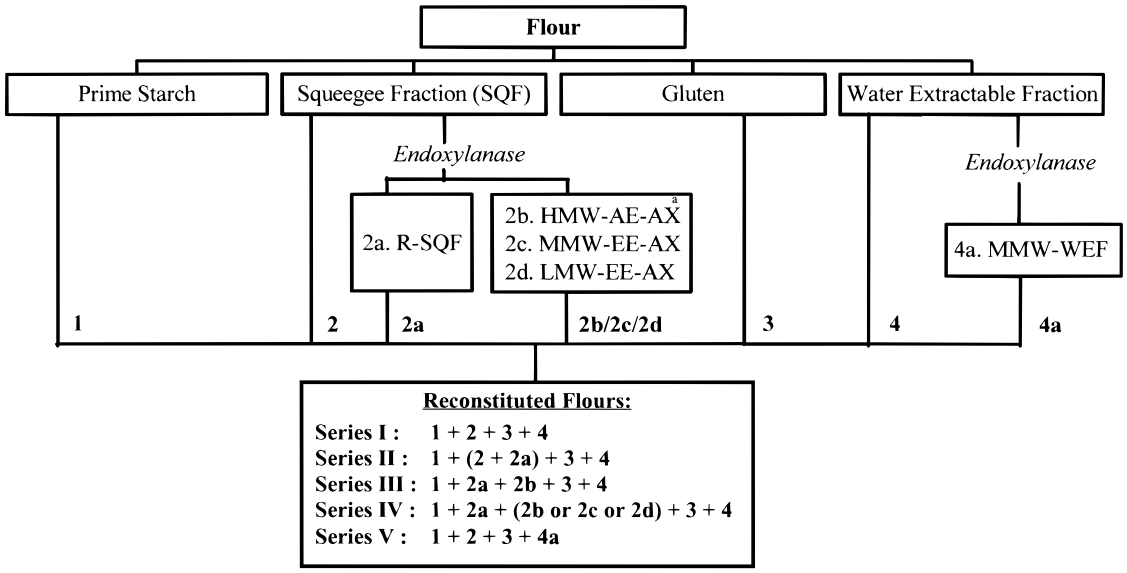

Figure 1. General overview of the experimental approach: series I, 1 + 2 + 3 + 4, reconstituted flour control; series II, 1 + (2 and 2a) + 3 + 4, reconstituted flours with varying WU-AX contents; series III, 1 + 2a + 2b + 3 + 4, reconstituted flours with WU-AX converted to nondegraded high molecular weight solubilized AX; series IV, 1 + 2a + (2b or 2c or 2d) + 3 + 4, reconstituted flours with solubilized AX degraded to diffferent MW; series V, 1 + 2 + 3 + 4a, reconstituted flours with WEF converted to MMW-WEF.

(15.0 g) of the Soissons and Torfrida SQFs and resulted in medium molecular weight enzyme-extracted AX (MMW-EE-AX) and residual SQF (R-SQF).

Further Enzymic Modification of MMW-EE-AX. MMW-EE-AXs of both Soissons and Torfrida (2.0 g) were dissolved in 100 mL of deionized water and incubated with 0.6 unit of endoxylanase (30 °C, 360 min). Hydrolysis was stopped by boiling the solution for 10 min. Following centrifugation (9000*g*, 10 min, 15 °C), the material was lyophilized. It will be further referred to as low molecular weight enzyme-extracted AX (LMW-EE-AX).

Enzymic Modification of WE-AX Present in the Water-Extractable Fractions. When the prime starch, gluten, SQF, and WEF are recombined, a flour is obtained that does not differ in functional (bread-making) properties from the starting material (Finney, 1943; MacRitchie, 1985). However, because the water-extractables also contain functional proteins (such as enzymes), we could not use a boiling step to inactivate endoxylanase used to degrade the WE-AX in this fraction. To overcome this inherent problem, we proceeded as follows: using the bread-making procedure of Shogren and Finney (1984), we first established an endoxylanase dosage−bread volume response curve with Soissons flour. For enzymic hydrolysis of WE-AX present in the water-extractable fraction, we then used an enzyme concentration 40 times lower than the optimal enzyme concentration for bread-making that, accordingly, had no impact on loaf volume.

Thus, WEF (3.0 g) was dissolved in 100 mL of water and 0.12 unit endoxylanase was added. The solution was incubated for 240 min at 30 °C, lyophilized, and passed through a 250 μm sieve. This material is further referred to as medium molecular weight water-extractable fraction (MMW-WEF).

Isolation and Purification of AX from SQF by Extraction with Base. Torfrida SQF (25.0 g) was suspended in 1000 mL of deionized water, and 0.9 Kilo Novo unit (KNU) of Thermamyl was added. After incubation (30 min, 75 °C), it was Büchner filtered. The residue was suspended in 0.4 KNU Thermamyl in 500 mL of water and incubated again. Amyloglucosidase (3000 units) was added, and the mixture was incubated for 12 h at 60 °C. Hydrolyzed starch was removed by dialysis (48 h, 4 °C). To the suspension obtained was added NaOH to a 0.1 M concentration. Extraction was overnight under continuous stirring at room temperature. After neutralization with HCl (0.1 M), the suspension was dialyzed for 36 h (4 °C). It was centrifuged (10000*g*, 15 min, 4 °C) and the supernatant lyophilized. This material was sieved through a 250 μm sieve and is further referred to as high molecular weight alkali-extracted AX (HMW-AE-AX).

Isolation and Purification of AX from the Water-Extractable Fraction of a Standard European Flour. The isolation was performed on the water-extractable fraction of a commercial, additive-free European bread-making flour and based on the method of Loosveld et al. (1997). Instead of subsequent drying of the WE-AX precipitate with ethanol and acetone washings, it was redissolved in deionized water and lyophilized. The obtained material, further referred to as HMW-WE-AX, was ground and sieved through a 250 μm sieve.

Gel Permeation Chromatography. Samples (1.0 mg) were solubilized in 0.3% NaCl and centrifuged (10000*g*, 10 min). Solutions obtained (20 μL) were separated on a Shodex SB-804 HQ GPC column (300 × 8 mm) with a Shodex SB-G guard column (50 × 6 mm) from Showa Denko K.K. (Tokyo, Japan). Elution was with 0.3% NaCl (0.5 mL/min at 30 °C) and a Kontron 325 pump system (Milan, Italy). 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 weights of 78.8×10^4, 40.4×10^4, 21.2×10^4, 11.2×10^4, 4.73×10^4, 2.28×10^4, 1.18×10^4, and 0.59×10^4 and glucose.

Reconstitution. Flour reconstitutions were based on the yields obtained in fractionation and modification steps, except for series II, in which substitution was on a weight basis.

A reconstitution was made with the originally obtained fractions to evaluate the fractionation−reconstitution procedure (Figure 1, series I).

The decrease in the amount of WU-AX caused by endoxylanase activity was simulated by stepwise substitution of SQF with R-SQF (Figure 1, series II). Reconstitution with complete substitution of SQF by R-SQF was called WU-AX 0.0. Reconstitution WU-AX 0.5 was made by substituting half of the original SQF with the same quantity of R-SQF. A reconstitution in which 1.5 times the amount of WU-AX originally present was incorporated (WU-AX 1.5) was made by using 1.5 times the normal amount of SQF. To this end, the original level of prime starch component was reduced accordingly.

The transformation of WU-AX to high molecular weight solubilized AX in regular bread-making with endoxylanase was simulated by substituting SQF with R-SQF and high molecular weight AX isolated from the SQF (HMW-AE-AX; Figure 1, series III) as well as from a standard wheat flour water-extractable fraction (HMW-WE-AX). Reconstitutions are des-

ignated WU-AX 0.0 + HMW-AE-AX and WU-AX 0.0 + HMW-EE-AX, respectively.

The degradation of the high molecular weight solubilized AX to lower molecular weight, as it may occur in regular bread-making with endoxylanase, was simulated by substituting SQF with R-SQF and the MMW-EE-AX or LMW-EE-AX (Figure 1, series IV). The former reconstitution is called WU-AX 0.0 + MMW-EE-AX and the latter WU-AX 0.0 + LMW-EE-AX.

Likewise, the degradation of the WE-AX by the enzyme in bread-making was modeled by substituting WEF with the enzyme-treated WEF (MMW-WEF). This reconstitution is called degraded WEF and is shown in Figure 1, series V.

Finally, bread was produced from reference reconstituted flour to which an optimal concentration of endoxylanase (0.72 unit) was added. The sodium chloride present in the enzyme-extracted AX samples was accounted for in bread-making.

Bread-Making. Wheat loaves (10 g) were produced in triplicate using the procedure of Shogren and Finney (1984). Doughs were mixed with a 10-g pin mixer (National Manufacturing, Lincoln, NE). Ingredients other than flour, yeast (Bruggeman, Brugge, Belgium), and water were sugar (sucrose, 6.0%) and salt (a total of 1.5%). Baking absorption was adjusted to a combination of optimum dough handling properties, loaf volume, and crumb structure, which was determined in several preliminary bread-making trails by an experienced baker. Due to the workload associated with fractionation and reconstitution and because the relevance of farinograph absorptions in experiments where modified AX is used is unknown, assessment of farinograph water absorption for the reconstituted doughs was omitted. Mixing time was kept constant, but for reconstituted doughs mixing was interrupted shortly after 1 min to incorporate material sticking to the sides of the mixing bowl. This typical behavior of reconstituted doughs is caused by rehydration phenomena and has been described earlier by Cawley (1964). Fermentation with fresh yeast (0.53 g) was 90 min, final proof was 35 min, and baking was 13 min at 232 °C. Volume readings were as described by Vanhamel et al. (1991).

RESULTS AND DISCUSSION

Chemical Composition of Native Flours and Their Fractions. Fractionation yields and analytical data of Soissons and Torfrida flours and flour fractions are presented in Table 1. Flour AX content is ~50% higher for Torfrida than for Soissons. Apart from natural variance, Torfrida's higher milling yield (74%) compared to that of Soissons (69%) might partially explain this observation because bran contains higher levels of AX than endosperm. As expected, both Soissons and Torfrida SQFs contained most of the WU-AX present in the respective flours, which led to their selection for further use in the WU-AX breakdown experiments. Gluten yields were as expected for European flours. A/X ratios for the water-extractable fractions were obtained by isolation of the WE-AX.

Enzymic Modification of WU-AX Present in the SQFs. *Preliminary Experiments.* Gel permeation chromatography profiles of endoxylanase solubilization of WU-AX present in the SQFs are shown in parts A and B of Figure 2 for Soissons and Torfrida flours, respectively. Partial chemical analysis of these fractions and apparent molecular weight data are given in Table 2. For Soissons SQF, the plateau of enzymic AX solubilization is reached sooner than for Torfrida. This is probably related to the higher WU-AX content in Torfrida flour, which requires longer incubation times to reach leveling off of AX solubilization, as well as to a lower accessibility of WU-AX of Torfrida flour because of a higher bran content (indicated by a higher ash content, Table 1).

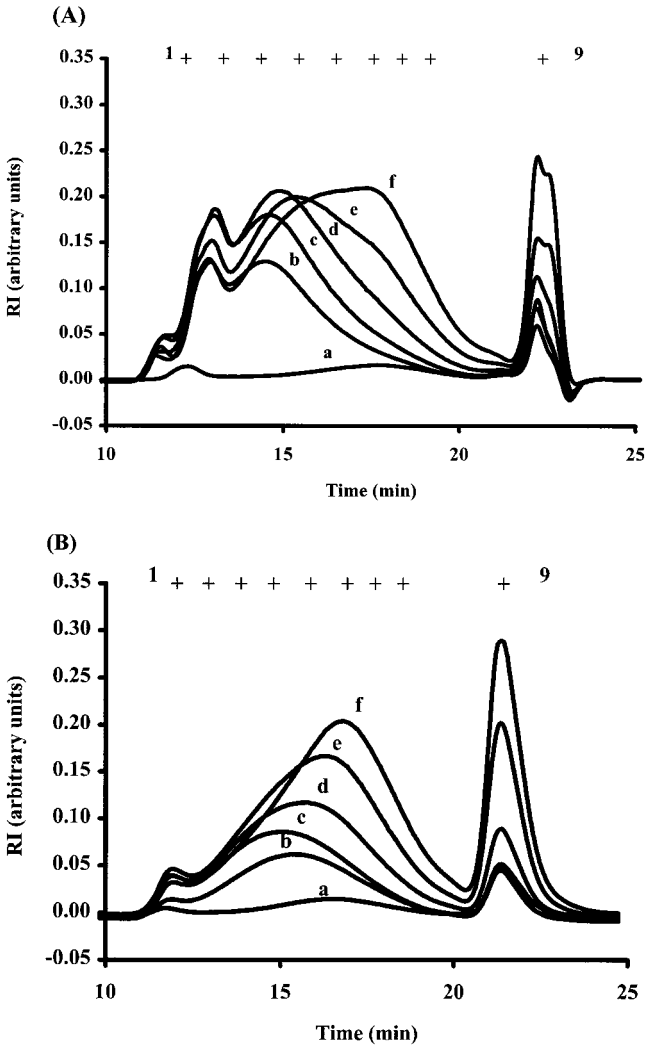

Figure 2. Molecular weight profiles of AX solubilized on incubation of **(**A**)** Soissons and **(**B**)** Torfrida SQF with *B. subtilis* endoxylanase for **(**a**)** 0 min, **(**b**)** 10 min, **(**c**)** 30 min, **(**d**)** 90 min, **(**e**)** 240 min, and **(**f**)** 480 min. Pullulan calibration standards are 78.8×10^4, 40.4×10^4, 21.2×10^4, 11.2×10^4, 4.73×10^4, 2.28×10^4, 1.18×10^4, 0.59×10^4, and glucose, numbers 1−9, respectively.

Of further note is the large difference in apparent weight-average MW ($MW_{app,wa}$) and apparent peak MW ($MW_{app,peak}$) between AX of both flours and this for all incubation times. These parameters are 2−3 times as high for Soissons as for Torfrida. Although one could suggest that the AX chains which build up the WU-AX are smaller for Torfrida than for Soissons, it seems more plausible that a difference in AX side-chain distribution influences susceptibility to enzymic attack. Lower enzyme activity toward AX chains containing more disubstituted xylose moieties (Coughlan and Hazelwood, 1993) may explain the observed results, as Soissons AXs have a much higher disubstituted to monosubstituted xylose ratio than Torfrida AXs (determined with ^{1}H NMR, results not shown).

Large-Scale Treatment. On the basis of the above, the large-scale incubation of SQF from Soissons and Torfrida flours (15.0 g) was carried out under identical conditions (with upscaling of the procedure) with incubation times of 90 and 240 min, respectively, to obtain as much WU-AX solubilization as possible without too much degradation of the enzyme-extracted AX polymeric structure. For Soissons, the resulting material

Table 2. WU-AX Contents (Percent), A/X of the WU-AX, and Molecular Weight Data of the Solubilized AX after Preliminary Breakdown Experiments of WU-AX from Soissons and Torfrida Flours

fraction	WU-AX[a]	A/X[b]	$MW_{app,peak}$[c]	$MW_{app,wa}$[c]
		(a) Soissons		
SQF	100.0	0.59	na[d]	na
SQF-control	97.9	0.58	24500	167000
SQF-10 min	70.3	0.61	250400	392900
SQF-30 min	48.0	0.62	233300	379500
SQF-90 min	35.2	0.66	184500	321800
SQF-240 min	29.7	0.64	132800	245000
SQF-480 min	29.5	0.61	33200	187500
		(b) Torfrida		
SQF	100.0	0.56	na	na
SQF-control	97.6	0.58	34800	78700
SQF-10 min	70.0	0.61	75500	129800
SQF-30 min	50.2	0.67	102500	161500
SQF-90 min	39.1	0.73	62600	132700
SQF-240 min	30.5	0.70	42000	105400
SQF-480 min	23.7	0.72	30200	80900

[a] WU-AX, ratio (%) of WU-AX remaining in SQF after enzymic treatment to the WU-AX originally present in SQF. [b] A/X = [Ara − (0.7 × Gal)]/Xyl]. [c] $MW_{app,peak}$, apparent peak MW; $MW_{app,wa}$, apparent weight-average MW. [d] na, not applicable.

Table 3. Molecular Weight Data of the Solubilized AX and WE-AX Fractions Obtained from Soissons and Torfrida Flours That Were Used for the Reconstitution Experiments

fraction	$MW_{app,peak}$[a]	$MW_{app,wa}$
	(a) Soissons	
HMW-WE-AX[b]	na[c]	na
HMW-AE-AX	na	na
MMW-EE-AX	184400	359700
LMW-EE-AX	18000	82600
WEF	>780000	na
MMW-WEF	244500	na
	(b) Torfrida	
HMW-WE-AX	>780000	501000
HMW-AE-AX	>780000	530000
MMW-EE-AX	107500	196100
LMW-EE-AX	22300	92200
WEF	>780000	na
MMW-WEF	295100	na

[a] $MW_{app,peak}$, apparent peak MW; $MW_{app,wa}$, apparent weight-average MW. [b] HMW-WE-AX, high molecular weight water extractable AX; HMW-AE-AX, high molecular weight alkali extracted AX; MMW-EE-AX, medium molecular weight enzyme extracted AX; LMW-EE-AX, low molecular weight enzyme extracted AX; WEF, water extractable fraction; MMW−WEF, medium molecular weight water extractable fraction. [c] na, not applicable.

was nearly identical to the one obtained in the above preliminary test. WU-AX content in R-SQF was 1.69% and A/X was 0.67. The complementary MMW-EE-AX material had an A/X of 0.56. Torfrida R-SQF material was somewhat different: the WU-AX content of 6.19% in the SQF was reduced to 1.28% (1.89% expected) in the R-SQF and A/X was 0.67 (instead of 0.70). Torfrida MMW-EE-AX had an A/X of 0.50. MW data are summarized in Table 3.

MMW-EE-AX from Soissons and Torfrida flours were further hydrolyzed to LMW-EE-AX. $MW_{app,peak}$ and $MW_{app,wa}$ are given in Table 3 and demonstrate that the further hydrolysis was effective. The chemical composition remained unchanged.

Enzymic Modification of WE-AX Present in WEF. Gel permeation profiles of WEF before and after enzymic hydrolysis are shown in parts A and B of Figure 3 for Soissons and Torfrida flours, respectively. Because

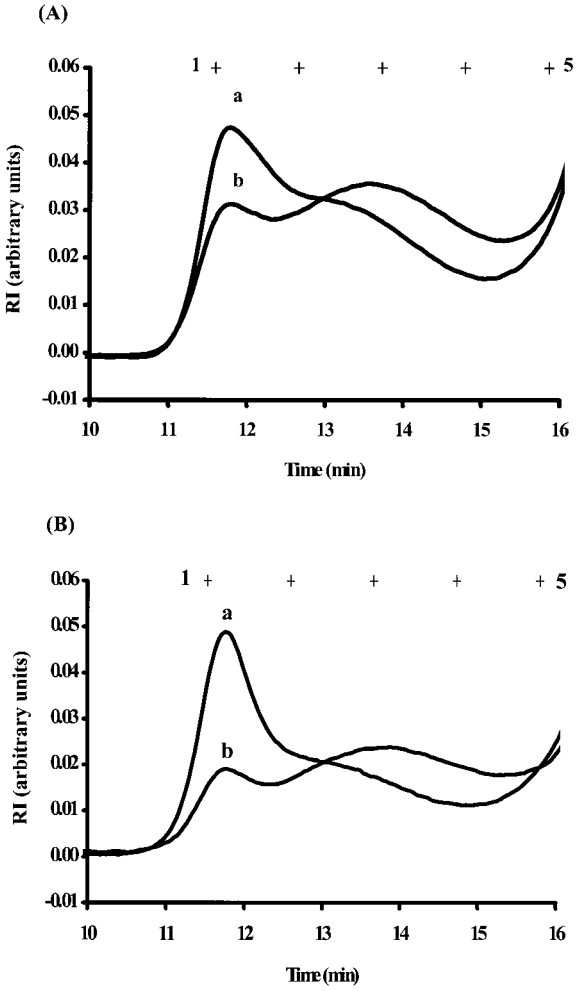

Figure 3. Partial molecular weight profiles of **(**A**)** Soissons and **(**B**)** Torfrida with **(**a**)** original water extractable fraction (WEF) and **(**b**)** water extractable fraction treated with *B. subtilis* endoxylanase for 240 min (MMW-WEF). Pullullan calibration standards are 78.8×10^4, 40.4×10^4, 21.2×10^4, 11.2×10^4, and 4.73×10^4, numbers 1−5, respectively.

of a very large non-AX mono- and oligosaccharide presence in the original and treated water-extractable fraction, only the high and medium MW region containing polymeric AX is shown. This yields qualitative information on AX breakdown. Approximate peak MW data are given in Table 3. It is clear that the incubation of the water-extractables with endoxylanase reduces the molecular weight of the components under study.

Composition of HMW-WE-AX and HMW-AE-AX Isolated from the Water-Extractables and SQF. The isolated HMW-WE-AX material contained 92.3% AX, 5.3% AGP, 0.3% starch, and virtually no protein. The corrected A/X ratio was 0.51. Molecular weight data are summarized in Table 3. The HMW-AE-AX isolate, after correction for the presence of sodium chloride, contained 92.1% AX with an A/X ratio of 0.54, 1.1% AGP, 5.1% polymeric glucose, and traces of protein.

Reconstitution and Bread-Making. Reconstitution schemes and the corresponding baking absorption and loaf volume results are displayed schematically in Figure 1 and Table 4.

Quality of Reconstitution: Series I. When the flour control is compared with the reconstituted flour control (Figure 1, series I), it is clear that both reconstitutions resulted in nearly identical loaf volumes, with no

Table 4. Effect of In Vitro Modification of AX Fractions on Baking Absorption (BA, Percent) and Specific Loaf Volume (Percent) for the European Wheat Flours Soissons and Torfrida

	(a) Soissons		(b) Torfrida	
reconstitution	BA	volume[b]	BA	volume
		Series I[a]		
native flour	55.8	102.4 ± 1.2	60.7	96.9 ± 1.4
reconstituted flour (ref)	55.8	100.0 ± 2.9	60.7	100.0 ± 1.2
		Series II		
WU-AX 0.0	51.5	113.1 ± 3.8	57.0	116.7 ± 2.0
WU-AX 0.5	53.5	106.0 ± 2.5	58.5	109.9 ± 2.5
WU-AX 1.0 (ref)	55.8	100.0 ± 2.9	60.7	100.0 ± 1.2
WU-AX 1.5	61.0	87.9 ± 2.2	65.0	85.6 ± 0.9
		Series III		
WU-AX 0.0 + HMW-WE-AX	53.5	132.8 ± 5.0	59.0	129.0 ± 0.6
WU-AX 0.0 + HMW-AE-AX	54.0	126.6 ± 2.1	60.0	129.1 ± 2.3
		Series IV		
WU-AX 0.0 + HMW-AE-AX	54.0	126.6 ± 2.1	60.0	129.1 ± 2.3
WU-AX 0.0 + MMW-EE-AX	53.0	132.2 ± 1.5	57.5	129.6 ± 3.7
WU-AX 0.0 + LMW-EE-AX	52.0	116.9 ± 2.3	55.5	106.6 ± 1.6
		Series V		
degraded WEF	55.0	89.7 ± 1.3	60.0	91.7 ± 1.2
reference + endoxylanase *(B.s.)*	55.0	128.3 ± 1.6	60.0	126.3 ± 2.3

[a] Series letters refer to the headings in the text under which the respective series is discussed. [b] Specific loaf volume is presented as the mean ± SD ($n = 3$) and is expressed as percentage of the reference volume (reconstituted flour). Reference loaf volumes for Soissons and Torfrida are 52 cm^3 and 50 cm^3, respectively.

statistically significant difference. For further discussion the reconstitution control is taken as reference.

Influence of Removal of WU-AX from Flour on Bread-Making: Series II. Gradual removal of WU-AX from flour has a significant effect on baking absorption (Table 4). A significantly positive linear correlation with $R^2 = 0.99$ for Soissons and $R^2 = 0.97$ for Torfrida ($P < 0.05$ for both) exists between WU-AX content and baking absorption. Taking into account the large water-holding capacity of the WU-AX in dough (Bushuk, 1966), this is no surprise. It is further evident that removal of WU-AX from flour is beneficial for the loaf volume of the resulting breads. A strong negative linear correlation was found between the two latter variables for both flours, with $R^2 = 0.92$ for Soissons and $R^2 = 0.96$ for Torfrida ($P \ll 0.05$ for both flours). These results are in agreement with early findings by Sandstedt et al. (1939) and by Maat et al. (1992) that loaf volume is correlated with the amount of tailings added to a dough. They furthermore confirm that this is due to the presence of WU-AX as also reported by Kulp and Bechtel (1963) and Krishnaray and Hoseney (1994). The latter studies furthermore indicated that, apart from loaf volume, also overall bread quality deteriorated upon addition of isolated WU-AX.

Influence of Converting WU-AX to Nondegraded Solubilized AX on Bread-Making: Series III. As enzymic treatment of the SQF showed that high molecular weight AX could not be obtained in this way, a next component of endoxylanase activity in bread-making, which is increasing the amount of nonhydrolyzed solubilized AX in dough, was simulated by substituting SQF with R-SQF and AX isolated from both SQF (WU-AX 0.0 + HMW-AE-AX) and the water-extractable fraction (WU-AX 0.0 + HMW-WE-AX). Both HMW-AE-AX and HMW-WE-AX were used to assess whether obvious differences exist between the functionality of AX isolated from WU-AX and WE-AX (Figure 1, series III). Upon substitution of WU-AX with nonhydrolyzed solubilized AX, baking absorption was higher than for the WU-AX 0.0 reconstitution (Table 4). However, it does not attain the level of the reference for either flour. This implies that the amount of water that has to be added to obtain optimal loaf volume is lower for AX in the soluble form than in the insoluble form. Table 4 further shows that the substitution adds another 19 and 12% volume increase to the 13 and 16% resulting from WU-AX removal from Soissons and Torfrida flours, respectively. This could be anticipated from a number of studies in which WE-AX or solubilized AX was added to flour. In most of these cases, bread loaf volume increases were found, together with significant increases in baking absorption (Cawley, 1964; Casier et al., 1973; Michniewics et al., 1992; Courtin and Delcour, 1998). WE-AX and alkali-solubilized AX seem to have the same effect when bread volume is concerned.

Influence of Hydrolysis of the Solubilized AX on Bread-Making: Series IV. Hydrolysis of the solubilized components by endoxylanase activity in dough was simulated as well. Reconstituted flours were made by substituting SQF with R-SQF and the MMW-EE-AX (WU-AX + MMW-EE-AX) or the LMW-EE-AX (WU-AX 0.0 + LMW-EE-AX) (Figure 1, series IV). Table 4 contains baking absorption and specific loaf volume values for the different combinations. Baking absorption is lowered progressively from WU-AX over HMW-AX and MMW-EE-AX to LMW-EE-AX. Rather surprisingly, bread-making with flour with mildly degraded MMW-EE-AX results in loaf volumes identical to those obtained with high molecular weight AX. Further hydrolysis of the MMW-EE-AX results in significant loss of loaf volume, although values for Soissons and Torfrida flours remain well above the data for the reference. If isolated HMW-WE-AX or HMW-AE-AX is a good substitute for nondegraded solubilized WU-AX, these data imply that there might be a threshold value above which variation in AX molecular weight is of little or no influence on loaf volume. Below that value, MW would positively relate with loaf volume. However, not enough data are present to substantiate this hypothesis.

When the results for reconstitutions WU-AX 0.0 and WU-AX 0.0 + LMW-EE-AX are compared in more detail, it becomes clear that, for Torfrida flour, complete removal of WU-AX is more beneficial for loaf volume

than hydrolyzing WU-AX to low molecular weight solubilized AX. At first glance, this observation seems somewhat contrary to an earlier observation by this group (Courtin and Delcour, 1998) that even low molecular weight AX addition gives loaf volume increases, albeit small. The result for Soissons flour does, however, not confirm this finding.

Influence of Hydrolysis of WEF to MMW-WEF on Bread-Making: Series V. Although baking absorption decreases only slightly, degradation of endogenous WEF to MMW-WEF (reconstitution degraded WEF) results in a significant drop of bread volume (Figure 1, series V; Table 4). Although the AX involved represents only an average 0.5% of flour weight and degradation is limited, loaf volume drops >8% below the reference value. The experiments by Cawley (1964) in which AX-degrading enzymes were used to hydrolyze the water-extractables gave similar results.

Finally, Table 4 lists the effect of adding an optimal amount of endoxylanase to the reconstituted flour. For Torfrida flour there is a significant difference between the maximal obtainable increase in loaf volume as represented by reconstitution WU-AX 0.0 + HMW-WE-AX (or WU-AX 0.0 + MMW-EE-AX) and the loaf volume increase induced by addition of endoxylanase. This is somewhat expected as endoxylanase has not only beneficial effects but also deleterious ones as outlined above. It thus seems logical that the potential loaf volume rise is more limited than found in our reconstitution experiments. Results for Soissons flour are similar, although loaf volume differences between in vitro and in vivo treated flours are not significantly different.

Previous experiments with Soissons flour (Courtin and Delcour, 1998) showed that adding HMW-WE-AX to flour at a 1.0% level, which corresponds with the amount of WU-AX present in Soissons flour, resulted in an increase in loaf volume of 10%. The results obtained here indicate not only that the increase in the amount of solubilized AX makes endoxylanases effective in increasing loaf volume but also that removal of WU-AX is equally relevant.

Insight. The above results allow us to hypothesise on the functional bread-making characteristics of AX. The main observation is that, in bread-making, WU-AX and WE-AX give rise to completely opposite effects. The difference here cannot be due to differences in the structure of the AX backbone, as Meuser and Suckow (1986) and Gruppen et al. (1992) indicated that those differences are small. Neither can they be due to differences in influence on baking absorption, as baking absorption changes only little when WU-AX is transformed into solubilized AX with high molecular weight. The largest difference between the two is simply that the one is soluble and the other is not. This implies that WU-AX cannot directly alter dough viscosity by entering into the dough liquor, but only indirectly by influencing the water distribution in the dough and trapping a (too) large amount of water. This contrasts with the WE-AX and solubilized AX, that, whether they are originally present as WE-AX, are added, or result from endoxylanase action, give rise to a considerable viscosity increase in the dough free water. This viscosity increase is, of course, dictated by molecular weight and by concentration, that is, the level of AX that moves into the dough liquor phase and may result in an optimum viscosity level of the dough at which bread-improvement will be optimal. This reasoning is in agreement with the findings of Cawley (1964), who considered viscosity to be the main factor determining functionality of WE-AX. Complementary to viscosity is the aspect of water redistribution. By solubilizing the WU-AX, the water retained is probably partially released and a redistribution over the different dough components is possible.

This brings us to a second aspect that applies for WE-AX and solubilized AX and not or much less for the insoluble ones, which is the possibility of interactions of AX with other AX or with other flour components. WE-AX or solubilized AX may, for example, give rise to a network by hydrogen bonds with itself, starch, and/or gluten and as such result in improved dough properties such as dough strength, gas retention, and stability during oven rise. Such interactions must be less probable for WU-AX, as they imply that insoluble material has to interact with insoluble material, which is less favored than interactions between insoluble and soluble or soluble and soluble material.

Conclusion. Enzymic in vitro modification of the WU-AX, the solubilized AX, and the WE-AX fractions of flour with an endoxylanase improved bread volume both by decreasing the amount of WU-AX in dough, on the one hand, and by increasing the amount of soluble AX, on the other. Degradation of WE-AX and extensive degradation of the solubilized AX decreased bread volume. This leads to the hypothesis that endoxylanases that have a bias toward WU-AX will perform well in bread-making and that endoxylanases with a bias toward WE-AX or solubilized AX, on the contrary, will not or only slightly influence loaf volume positively. The results further suggest that WE-AX and solubilized AX are beneficial for bread-making because of their soluble nature and the consequences thereof for dough characteristics. WU-AX could be deleterious for loaf volume because of their insolubility and resulting water immobilization.

ABBREVIATIONS USED

AGP, arabinogalactan peptide; AX, arabinoxylan; A/X, corrected arabinose-to-xylose ratio; dm, dry matter; HMW-WE-AX, high molecular weight water extractable AX; HMW-AE-AX, high molecular weight alkali extracted AX; LMW-EE-AX, low molecular weight enzyme extracted AX; MMW-EE-AX, medium molecular weight enzyme extracted AX; MMW-WEF, medium molecular weight water extractable fraction; R-SQF, residual squeegee fraction; SQF, squeegee fraction; WE-AX, water extractable AX; WEF, water extractable fraction; WU-AX, water unextractable AX.

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