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Impact of Xylanases with Different Substrate Selectivity on Gluten−Starch Separation of Wheat Flour

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The influence on wheat flour gluten−starch separation of a xylanase from *Aspergillus aculeatus* (XAA) with hydrolysis selectivity toward water extractable arabinoxylan (WE-AX) and that is not inhibited by wheat flour xylanase inhibitors was compared to that of a xylanase from *Bacillus subtilis* (XBS) with hydrolysis selectivity toward water unextractable arabinoxylan (WU-AX) and that is inhibited by such inhibitors. XAA improved gluten agglomeration through degradation of WE-AX and concomitant reduction in viscosity, which in the laboratory scale batter procedure with a set of vibrating sieves (400, 250, and 125 μm), increased protein recoveries on the 400 μm sieve. In contrast, XBS had a negative effect as it decreased gluten protein recovery on this sieve, probably as a result of the viscosity increase that accompanied WU-AX solubilization. Hence, it was active even if most likely a considerable part of its activity was prevented by xylanase inhibitors. A combination of XAA and XBS at a low dosage yielded a distribution of gluten proteins on the different sieves comparable to that of the control. At a high combined dosage, the gluten agglomeration was better than that with XAA alone, indicating that both WE-AX and WU-AX have a negative impact on gluten agglomeration. Finally, experiments with endoxylanase addition at different moments during the separation process suggest that the status of the arabinoxylan population during dough mixing is far less critical for its impact on gluten agglomeration than that during the batter phase.

KEYWORDS: Wheat flour; xylanase; arabinoxylans; gluten agglomeration

INTRODUCTION

Separation of wheat flour into vital gluten and starch is an important industrial process. Several aspects determine the efficiency of the separation and the yield and quality of the end products. Apart from the concentration and properties of the gluten proteins in wheat flour, AX can also influence gluten agglomeration (*1*−*3*). Such AX are present either as WE-AX or as WU-AX. These two AX populations have different physicochemical properties and play an important role in the gluten−starch separation. WE-AX excel in viscosity-forming potential (*4*, *5*), and WU-AXs excel in water-holding capacity (*6*).

The structure and properties of the AX populations can be strongly influenced by xylanases that solubilize WU-AX and degrade WE-AX and S-AX to molecules of lower molecular weight (*7*, *8*). An enzyme mixture containing hemicellulase improves gluten agglomeration behavior as well as starch yield of a flour with intermediate processing properties (*9*). The role of xylanases in this separation process has been ascribed to their effect on viscosity (*10*). A reduced viscosity of a wheat flour slurry improves gluten agglomeration (*11*, *12*).

Xylanase inhibitors like TAXI (*13*, *14*) and XIP (*15*) can change the activity of commonly used xylanases. These inhibitors can hence affect the functionality of xylanases in gluten−starch separation. No scientific studies have been done on the impact of inhibition in this process.

The objective of the present study was to gain insight in the influence of xylanases and of wheat flour AX on gluten agglomeration in the gluten−starch separation. To this end, xylanases with different hydrolysis selectivity toward WE-AX and WU-AX and different xylanase inhibitor sensitivity were added in different dosages and at different moments during the separation process in a laboratory scale dough batter procedure. The gluten agglomeration behavior, the AX population, and the viscosity development during the separation were studied, and the impact of substrate selectivity and inhibition on the xylanase functionality in gluten−starch separation was assessed. The carbohydrate compositions of the gluten samples were determined. We here report on the outcome of this work.

MATERIALS AND METHODS

Materials. XAA (Shearzyme 500L, Novo Nordisk, Bagsvaerd, Denmark) and XBS (Grindamyl H640, Danisco, Brabrand, Denmark) were added during gluten−starch separation. They were chosen because of their different substrate selectivity and inhibitor sensitivity. XBS has a greater selectivity for WU-AX, whereas XAA rather degrades WE-AX and to a more limited extent solubilizes WU-AX (*8*). Indeed, substrate selectivity factors, i.e., the ratios of enzyme solubilizing

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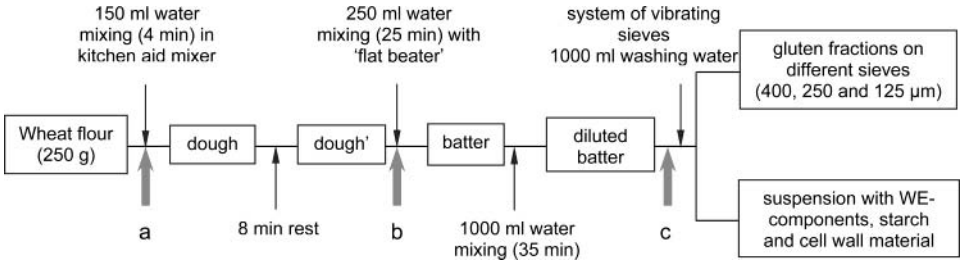

Figure 1. Scheme of the batter procedure for wheat flour fractionation with xylanase addition at different stages of the process (indicated with the gray arrow). Trial a, xylanase added to the flour at the beginning of the dough mixing step; trial b, xylanase added to the dough after resting; trial c, xylanase added to the batter 10 min before the end of the batter phase of the process.

activity toward insoluble substrate over enzyme hydrolyzing activity toward soluble substrate, of XBS and XAA are 7.2 and 0.9, respectively (*16*). XBS is inhibited by TAXI proteins, whereas XAA is insensitive for inhibition by TAXI (*13*). Neither XBS nor XAA are inhibited by XIP (*15*).

One unit of xylanase activity (U) is defined as the volume (μL) of enzyme solution needed to yield a change in extinction (590 nm) of 1.0 at pH 6.0 in the AZCL-AX procedure, described in the Megazyme Data Sheet 9/95 (Bray, Ireland).

After conditioning to 16.0% moisture, wheat (variety Zohra, moderate bread-making quality, Aveve, Landen, Belgium) was milled on a Bühler MLU-202 laboratory mill (Uzwill, Switzerland) according to the AACC method (*17*). The resulting flour had a protein content of 10.1% (*18*) and a Farinograph water absorption of 58.7% (14.0% moisture base) (*17*). The flour contained 0.38% WE-AX and 1.63% WU-AX, determined with gas chromatography as described further.

To evaluate to what extent XBS was potentially inhibited by flour xylanase inhibitors during processing, the xylanase inhibitor activity of Zohra wheat flour was determined with an assay (*13*) in which wheat water extract was incubated with xylanase prior to assessment of residual xylanase activity. The highest XBS dosage (1.60 U/g flour) used during flour fractionation was added to a wheat flour extract in the same proportion as during the fractionation process. Under these conditions, the inhibitors of the flour extract decreased xylanase activity by 89%. This test was not performed for XAA since this xylanase was inhibited by neither TAXI nor XIP proteins.

Wheat Flour Fractionation with or without Xylanase Addition at the Beginning of Mixing. The laboratory scale wheat flour fractionation procedure was described by Frederix et al. (*19*) and was based on pilot scale batter procedures (*20*−*23*). Wheat flour (250 g, 14.0% moisture base) was mixed with water (150 mL, Farinograph water absorption) in a KitchenAid mixer (K5SS/KPM5, St. Joseph, MI) equipped with a dough hook (4 min). XAA and XBS were added to the flour at the beginning of mixing in different dosages ranging from 0 to 0.56 U/g flour for XAA and from 0 to 1.60 U/g flour for XBS. Combinations of both enzymes were also added to wheat flour at two different dosages: an intermediate level (0.14 U XAA and 0.80 U XBS/g flour) and a very high dosage (5.6 U XAA and 16 U XBS/g flour). The enzymes were dissolved in the flour mixing water. The dough was allowed to rest for 8 min. Additional water (250 mL) was added, and the suspension was stirred with a flat beater for 25 min. After extra water (1000 mL) was added, the suspension was further stirred for 35 min. The suspension was brought over vibrating sieves with decreasing pore size (400, 250, and 125 μm). The gluten on the vibrating sieves was washed with extra water (1000 mL). Gluten fractions were recovered from the three sieves, lyophilized, and ground to pass a 250 μm sieve.

Dough samples were withdrawn after mixing and after resting. A sample was equally taken from the water extractable fraction of the batter right before sieving through centrifugation of the batter (5000*g*, 15 min, 4 °C). These samples were immediately frozen in liquid nitrogen and lyophilized.

Wheat Flour Fractionation with or without Xylanase Addition at Different Stages of the Process. In further trials, xylanase (0.28 U XAA/g flour or 0.80 U XBS/g flour) was added to the dough after resting or to the batter 10 min before the end of the batter phase of the process, instead of to the flour at the beginning of mixing (**Figure 1**).

Analysis of Protein Contents. Protein contents were determined using an adaptation of the AOAC Official Dumas Method to an automated Dumas protein analysis system (EAS varioMax N/CN, Elt, Gouda, The Netherlands) (*18*). The coefficient of variation of the analysis was less than 1%.

Definitions (*23*) are used as follows: (i) GPR_{xxx}, i.e., g of protein (dm) agglomerated on the sieve with a pore diameter of xxx μm from 100 g of flour protein (dm). (ii) GPR_{tot}, i.e., the total amount of protein (dm) agglomerated on the three consecutive sieves (400, 250, and 125 μm) per 100 g of flour protein (dm). The coefficient of variation of the GPR was less than 5, 15, and 10% for the 400, 250, and 125 μm sieves, respectively. The GPR and the distribution of proteins over the different sieves were used as a measure for the agglomeration behavior and reflect the processing properties of the flour. (iii) GPAI, i.e., the ratio (in percent) of protein agglomerated on the 400 μm sieve to the total amount of protein recovered from the three consecutive sieves. This index was used as a measure for the tendency of gluten to agglomerate and form large gluten aggregates.

Extraction of Flour and Dough Samples. Flour and dough samples were extracted as described earlier (*24*). Lyophilized dough samples (5.00 g) were extracted with deionized water (100 mL, 4 °C) for 30 min at 4 °C. After the samples were centrifuged (10 000*g*, 15 min, 4 °C), the supernatant was immediately frozen with liquid nitrogen. The residue was washed with water (50 mL, 4 °C) and centrifuged again. This supernatant was also immediately frozen as above. After the samples were lyophilized, boiling water (150 mL) was added to inactivate the enzyme (30 min, 100 °C). After renewed lyophilization, the material was dispersed in water (50 mL) and centrifuged (10 000*g*, 15 min, 4 °C), and the supernatant was analyzed for carbohydrate composition, molecular weight distribution, and viscosity. The same inactivation procedure was followed for the sample of the water extractable fraction of the batter taken during the fractionation procedure. Here, the extraction step was skipped since the sample of the batter had already been centrifuged and lyophilized.

Analysis of Carbohydrate Composition. The carbohydrate compositions of the dough and batter extracts and the gluten fractions were determined by gas chromatography of alditol acetates obtained after acid hydrolysis, reduction, and acetylation of the samples (*25*). The samples were separated on a Supelco SP-2380 polar column (Supelco, Bellefonte, PA) in an Agilent chromatograph (Agilent 6890 series, Wilmington, DE) equipped with a flame ionization detector. The inlet and detector temperatures were 270 °C, while separation took place at 225 °C. The carrier gas was He. The coefficient of variation of the analysis was less than 5%.

HPSEC. Dough and batter extracts were separated on a Shodex SB-806 HQ HPSEC column with a molecular weight range of 100−20 000 000 (Showa Denko K. K., Tokyo, Japan). Elution was with 0.3% NaCl (0.5 mL/min at 30 °C), and monitoring was with a refractive index detector (VSD Optilab, Berlin, Germany). Molecular weight markers were glucose and Shodex standard P-82 pullulans with molecular weights of 788×10^3, 404×10^3, 212×10^3, 112×10^3, 47.3×10^3, 22.8×10^3, 11.8×10^3, and 5.6×10^3.

Measurement of Viscosity. The dough and batter extract viscosities (30 °C) were measured with an Ostwald viscometer according to Vinkx et al. (*26*). The viscosity was expressed relative to that of water under the same conditions. The coefficient of variation of the measurement was less than 2%.

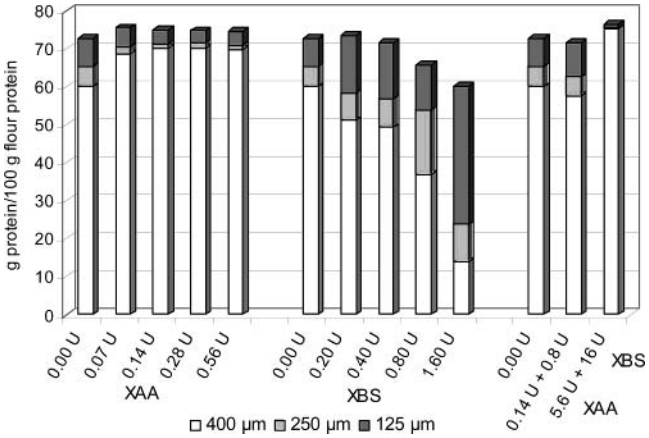

Figure 2. Gluten protein recoveries on the 400, 250, and 125 μm sieves with different dosages (U/g flour) of XAA and XBS added at the beginning of the dough mixing step in the gluten–starch separation procedure.

RESULTS

Gluten Agglomeration Behavior with Xylanase Addition at the Beginning of Mixing. Figure 2 shows the distribution of the gluten proteins over the different sieves for the addition of different dosages of XAA and XBS at the beginning of the process (dough mixing stage). XAA improved the agglomeration behavior of the gluten proteins over the control. The GPR_{400} increased from 60 to 70 g proteins/100 g flour proteins with increasing enzyme dosages and then reached a maximum. Simultaneously, GPR_{250} and GPR_{125} values decreased. GPR_{tot} was only slightly influenced by the addition of XAA. As a consequence, GPAI, a good measure for the gluten agglomeration behavior and the formation of larger gluten aggregates, increased from 83% for 0 U XAA to 93% for 0.14 U XAA/g flour to then approximately remain constant indicating that with this enzyme, the gluten agglomeration behavior could not be further improved. The above is consistent with the finding (*9*) that hemicellulase improves gluten agglomeration and with the positive impact of a pure XAA in gluten−starch separation (*10*, *11*).

XBS decreased the GPR_{400} from 60 to 14 when the enzyme dosage increased from 0 to 1.60 U/g flour (**Figure 2**). This coincided with a decrease in GPR_{tot} from 73 to 60. The decrease in GPAI from 83 to 23% indicates that the level of large gluten aggregates drastically decreased and that more gluten proteins were retained on the 250 and 125 μm sieves.

The gluten protein agglomeration behavior upon addition of both XAA (0.14 U/g flour) and XBS (0.80 U/g flour) together was similar to that of the control without xylanase addition. Apparently, XAA neutralized the effect of XBS. At these intermediate XAA and XBS dosages, a very small decrease in GPR_{tot} and GPAI was observed. However, the simultaneous addition of high dosages of XAA (5.6 U/g flour) and XBS (16 U/g flour) improved the gluten agglomeration, even more than with the addition of XAA alone. The GPR_{400} increased from 60 (control) to 75. This coincided with an increase in GPR_{tot} from 73 to 77. The GPAI clearly indicated an improved agglomeration behavior as it increased from 83 to 98%. At these high XAA and XBS dosages, approximately all gluten proteins were retained on the 400 μm sieve. Apparently, the xylanase action increased gluten tendency or capability to form large gluten aggregates. As the joint use of XBS and XAA improved gluten agglomeration more than XAA alone, this strongly suggests that both WU-AX (the preferred substrate of XBS) and WE-AX (the preferred substrate of XAA) impact the gluten−starch separation process.

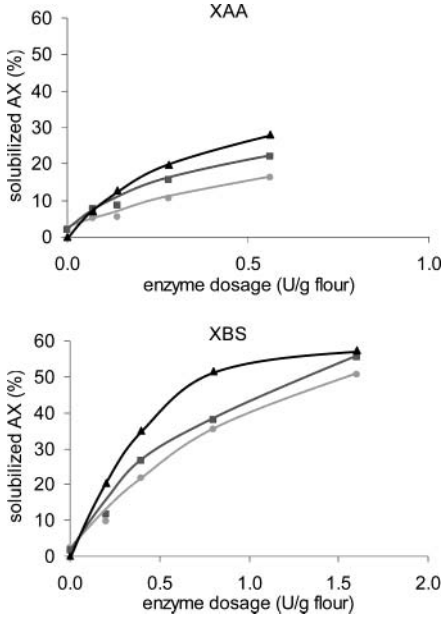

Figure 3. Solubilization of WU-AX (ratio of solubilized AX to original WU-AX) as a function of XAA and XBS dosage (●, dough after mixing; ■, dough after rest; ▲, batter). Addition of XAA and XBS at the beginning of the dough mixing step in the gluten–starch separation procedure.

Properties of AX in Dough and Batter with Xylanase Addition at the Beginning of Mixing. *Solubilization of WU-AX and Impact of Xylanase Inhibitors.* With increasing XAA dosages, solubilization of WU-AX increased from 2 (0 U XAA) to 16% (0.56 U XAA) during dough mixing and from an additional 0 (0 U XAA) to 6% (0.56 U XAA) during dough resting (**Figure 3**). During the batter phase, solubilization of WU-AX further continued, and at the end of the process, 28% of WU-AX was solubilized at a XAA dosage of 0.56 U/g flour.

The solubilization of WU-AX increased from 2 to 50% with increasing XBS dosages during dough mixing. During dough resting and the batter phase, additional solubilization varied between 0 and 5% and between 0 and 14%, respectively. XBS solubilized most of the WU-AX during dough mixing. At the end of the batter phase, a solubilization of 57% was reached for 1.60 U XBS/g flour. At this XBS level, only a very small part of the WU-AX (2%) was solubilized during the batter phase of the process. Apparently, the solubilization had reached its upper limit. It seems probable that TAXI proteins had inactivated XBS (*13*). Inhibitor activity measurements on Zohra wheat flour water extracts indeed indicated that with the addition of the highest XBS dosage (1.60 U/g flour), the inhibitors present in the flour were able to inhibit 89% of the xylanase activity added. While the inhibitors theoretically must have been able to completely inhibit the lower levels of XBS added, their effects on the process suggest a progressive loss of activity with process time.

With the addition of both XAA (0.14 U/g flour) and XBS (0.80 U/g flour), 58% of the WU-AX was solubilized at the end of the batter phase. At high XAA and XBS dosages (5.6 U XAA and 16 U XBS/g flour), 80% of WU-AX was solubilized. These high solubilization degrees are similar to those found earlier (*7*, *24*).

Molecular Weight Profiles. Addition of XAA degraded the high molecular weight WE-AX (>200 000) to lower molecular weight fragments (**Figure 4**). The level of AX fragments with a molecular weight between 11 000 and 150 000 increased with enzyme dosage. XBS solubilized WU-AX yielding high molecular weight S-AX. At an enzyme dosage of 1.60 U/g wheat

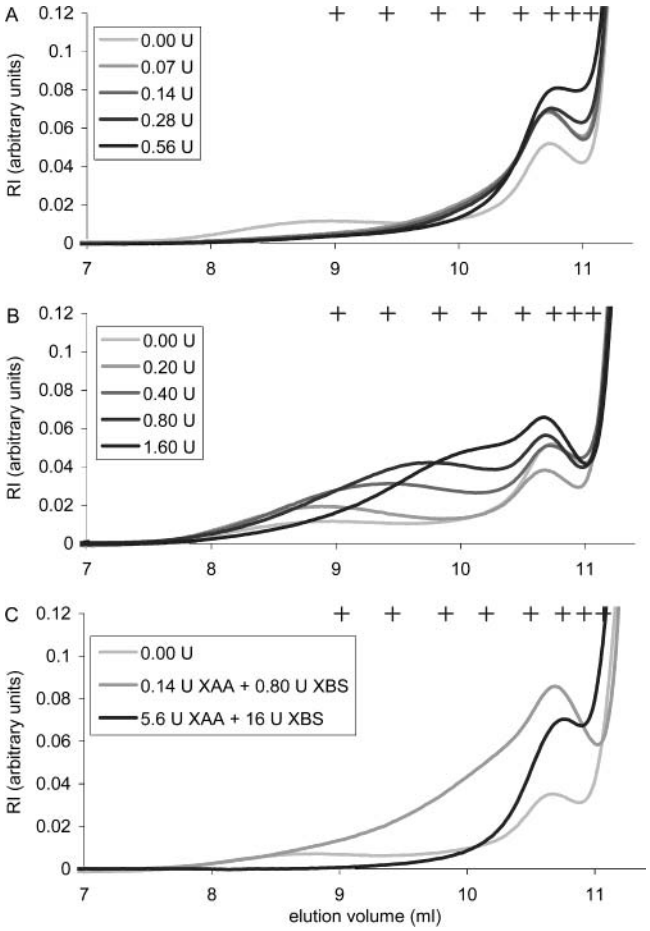

Figure 4. HPSEC molecular weight profiles of the water extractable fractions of the batters with increasing xylanase dosages added at the beginning of the dough mixing step in the gluten–starch separation procedure. (**A**) XAA, (**B**) XBS, and (**C**) XAA + XBS. MW markers from left to right: 788, 404, 212, 112, 47.3, 22.8, 11.8, and 5.9×10^3.

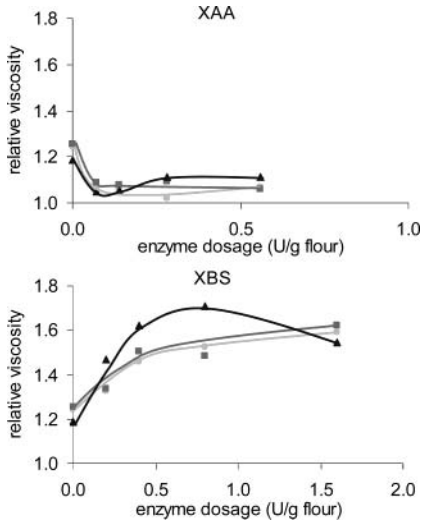

Figure 5. Relative viscosity of dough and batter extracts as a function of XAA and XBS dosage (●, dough after mixing; ■, dough after rest; ▲, batter). Addition of XAA and XBS at the beginning of the dough mixing step in the gluten–starch separation procedure.

flour, degradation of S-AX became more evident and the molecular weight profiles shifted toward lower molecular weights. The profiles of samples where XAA (0.14 U/g flour) and XBS (0.80 U/g flour) were added together showed both a strong solubilization and a degradation. XBS mainly solubilized WU-AX while XAA degraded the high molecular weight WE-AX and S-AX. At high dosage levels (5.6 U XAA and 16 U XBS/g flour), the high molecular weight WE-AX and S-AX were efficiently degraded to low molecular weight fragments.

Relative Viscosity. The viscosity of dough and batter extracts is mainly caused by wheat flour AX (*4*). From this and the xylanase specificity, it follows that the change in viscosity of dough and batter extracts results from changes in the AX population such as the solubilization of WU-AX, which increases S-AX levels and the changes in the molecular weight of WE-AX and S-AX. These parameters mainly determine the viscosity of the extracts.

Increasing XAA dosages decreased the relative viscosities of dough and batter extracts because of the (preferential) degradation of WE-AX (**Figure 5**). XBS solubilized high levels of WU-AX and concomitantly increased the concentration of S-AX and hence the viscosity of dough and batter extracts. While a XBS dosage of 1.60 U/g wheat flour increased the viscosity of the dough extracts, that of the resulting batters decreased. At this XBS dosage, solubilization had reached its upper limit and degradation of WE-AX and S-AX increased with process time.

Combined, the solubilization, HPSEC, and viscosity profiles (**Figures 3**−**5**) clearly show the difference in mode of action of XAA and XBS. At the same degree of solubilization, the molecular weight profiles and viscosities of dough and batter extracts were completely different. XBS has a greater selectivity toward WU-AX, whereas XAA rather degrades WE-AX and, to a more limited extent, solubilizes WU-AX. Besides their different substrate selectivity, the inhibition of XBS by TAXI proteins and the insensitivity of XAA for inhibition by TAXI can also be an important factor determining their different solubilization and degradation behavior.

A combination of XAA (0.14 U/g flour) and XBS (0.80 U/g flour) solubilized 58% of WU-AX during the separation. However, the viscosity at the end of the batter phase was only slightly higher than that of the control without enzyme addition because of S-AX and WE-AX degradation to lower molecular weight fragments. High dosages of XAA (5.6 U/g flour) and XBS (16 U/g flour) together solubilized 80% of the WU-AX, but the degradation of S-AX and WE-AX was responsible for the low relative viscosity of the batter (1.02).

Xylanase Addition at Different Stages of the Process. In the next phase, we added xylanases at different moments during the separation process to better understand at which point the AX population impacts the gluten agglomeration behavior. Because the viscosity of the batter extract is mainly caused by the AX, this experiment can also elucidate during which phase of the procedure viscosity is important for the separation behavior of wheat flour.

Figure 6A shows the distribution of gluten proteins on the sieves when adding 0.28 U XAA/g flour or 0.80 U XBS/g flour at the beginning of flour mixing, to the dough after resting and to the batter 10 min before the end of the batter phase.

The addition of xylanases to the dough after resting instead of prior to mixing resulted in a similar distribution of gluten proteins on the sieves. The effect of xylanases during mixing and resting of the dough is therefore not that determining for the distribution of the gluten proteins on the different sieves later in the process. This is rather surprising since the gluten network is developed during dough mixing. When added at the

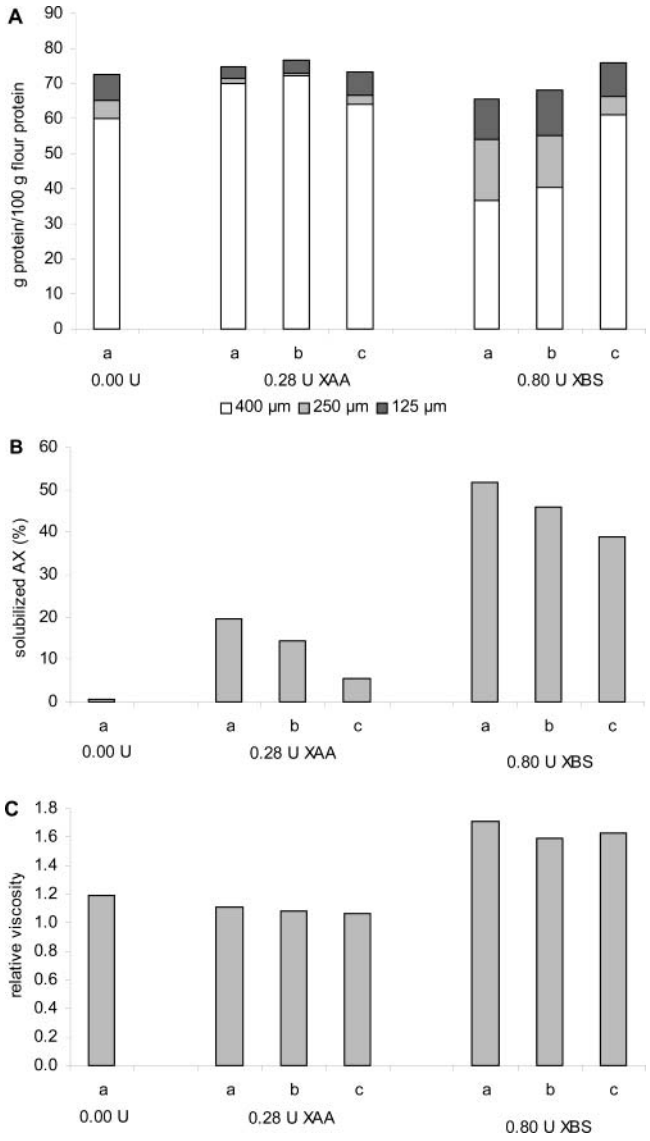

Figure 6. Gluten protein recoveries on the 400, 250, and 125 μm sieves (**A**), solubilization of WU-AX (ratio of solubilized AX to original WU-AX) (**B**), and relative viscosity of the batter (**C**) for the addition of 0.28 U XAA/g wheat flour or 0.80 U XBS/g wheat flour at different moments during the separation process. (a) Xylanase added to the flour at the beginning of the dough mixing step; (b) xylanase added to the dough after resting; and (c) xylanase added to the batter 10 min before the end of the batter phase of the process.

beginning of the process, xylanases change the AX population of wheat flour already during mixing. The solubilization of WU-AX and degradation of WE-AX and S-AX influence the distribution of water in the dough and change the environment for gluten development and hydration.

The distribution of gluten proteins with xylanase addition 10 min before the end of the batter phase was approximately the same as in the control without xylanase addition. The xylanase action during these last 10 min of the batter phase had no clear influence on gluten agglomeration, although the AX population was (seriously) affected.

Next to gluten protein distribution, **Figure 6** also shows WU-AX solubilization and batter viscosities resulting from the addition of 0.28 U XAA/g wheat flour or 0.80 U XBS/g wheat flour at different moments in the separation process. The solubilization of WU-AX decreased when the xylanase was added later in the process. Surprisingly, with addition of xylanase to the dough after resting and at 10 min before the end of the batter phase, the viscosity of the batter differed only slightly from that with addition of xylanase to flour.

Carbohydrate Composition of the Gluten Fractions. Table 1 shows the carbohydrate composition of the gluten fractions with addition of different XAA and XBS dosages at the beginning of mixing. Besides glucose, which is quantitatively the most important monosaccharide, the gluten fractions also contained arabinose, xylose, and galactose. Arabinose is present as a building block of both AX and arabinogalactan peptides, while galactose is mainly part of arabinogalactan peptides and galactolipids. The exact calculation of the AX content of the gluten samples is not possible because it is unclear how much arabinose in gluten originates from AX and how much from arabinogalactan peptides.

The arabinose content of the 400 μm gluten fraction varied between 0.36 and 0.58%, and the xylose content varied between 0.31 and 0.57%. There was no clear influence of xylanase addition on the AX content of the 400 μm gluten fractions, and no direct correlation was found between the arabinose and xylose contents and the GPR_{400}.

The AX contents of the gluten fractions increased as the pore diameter of the sieve decreased. This is in agreement with the results of Roels et al. (*27*). The arabinose and xylose levels in the 250 μm gluten fraction ranged from 1.1 to 2.6% and from 1.2 to 3.0%, respectively, except for the 250 μm gluten fraction obtained with the addition of high levels of both XAA and XBS (5.6 U XAA and 16 U XBS/g flour). The latter contained 6.5% arabinose and 7.4% xylose. Here, the very small amount of material recovered from the 250 μm sieve should be considered.

With different dosages of XAA, the 125 μm sieve gluten fraction contained between 4.4 and 8.4% arabinose and between 6.4 and 12.7% xylose. XBS decreased arabinose and xylose contents of the 125 μm gluten fractions from 5.7 to 1.9% and from 9.0 to 2.6%, respectively. The galactose contents of the gluten fractions fluctuated between 0.67 and 1.12% and are similar to data of Roels et al. (*27*) for gluten fractions isolated from six different wheat varieties.

The glucose contents of the 400 μm gluten fraction ranged from 15.7 to 25.2%. An increased GPR_{400} coincided with an increased glucose content of the 400 μm gluten fraction (correlation coefficient = 0.83). The improved agglomeration behavior is probably responsible for an increased amount of starch entrapped in the gluten matrix, as also indicated by Wang et al. (*2*). The glucose content of the gluten fraction retained on the 250 μm sieve was smaller than that of the 400 μm gluten fraction, whereas the 125 μm gluten fraction again contained an increased glucose level.

Figure 7 shows the linear relation between GPR_{400} and the levels of arabinose and xylose associated with the gluten fraction retained on the 400 μm sieve (g monosaccharide/100 g flour monosaccharide). In analogy with the GPR terminology, these levels are further referred to as ara-R and xyl-R. The ara-R and xyl-R express the percentages of arabinose and xylose originally present in wheat flour associated with the 400 μm gluten fraction. For arabinose, this level varied between 0.8 and 4.8%, whereas between 0.5 and 3.2% of the flour xylose was associated with the 400 μm gluten fraction. Ara-R is probably higher than xyl-R because of the interaction of arabinogalactan peptides in addition to AX with the gluten. The higher the GPR_{400}, the more arabinose and xylose originally present in wheat flour associated with the gluten proteins. This is mainly a result of an increased gluten yield on the 400 μm sieve rather than an increased AX concentration of the gluten fractions. Roels

Table 1. Carbohydrate Composition[a] (% w/w, dm) of the Gluten Fractions (400, 250, and 125 μm Sieve) with the Addition of Different XAA and XBS Dosages at the Beginning of Mixing

	400 μm				250 μm				125 μm			
	ara	xyl	gal	glc	ara	xyl	gal	glc	ara	xyl	gal	glc
						control						
0.00 U	0.47	0.46	0.95	20.80	1.10	1.26	1.00	14.62	5.67	9.02	0.93	26.44
						XAA						
0.07 U XAA	0.46	0.43	0.95	22.64	2.26	2.76	1.02	15.46	5.64	8.66	0.87	32.51
0.14 U XAA	0.47	0.45	0.95	24.05	1.18	1.27	1.02	11.21	4.36	6.42	0.67	47.15
0.28 U XAA	0.43	0.43	0.89	24.73	1.46	1.61	1.05	11.42	8.36	12.72	1.02	23.70
0.56 U XAA	0.43	0.42	0.90	25.20	2.62	3.02	1.02	16.25	6.42	9.45	1.02	19.92
						XBS						
0.20 U XBS	0.41	0.37	0.96	18.70	1.21	1.40	0.98	17.48	3.55	5.33	0.92	24.55
0.40 U XBS	0.50	0.47	0.96	19.00	1.39	1.65	1.01	15.68	4.08	6.04	0.93	23.27
0.80 U XBS	0.58	0.57	1.01	16.40	1.73	2.17	1.07	15.21	2.36	3.34	1.01	16.33
1.60 U XBS	0.43	0.39	0.94	15.68	1.10	1.24	1.05	14.63	1.89	2.57	1.00	17.95
						XAA + XBS						
0.14 U XAA + 0.80 U XBS	0.42	0.35	1.00	19.61	1.97	2.39	1.03	18.70	4.15	5.97	1.12	18.31
5.6 U XAA + 16 U XBS	0.36	0.31	0.90	22.40	6.45	7.39	1.11	15.10	8.72	10.95	1.12	32.63

[a] ara, arabinose; xyl, xylose; gal, galactose; and glc, glucose.

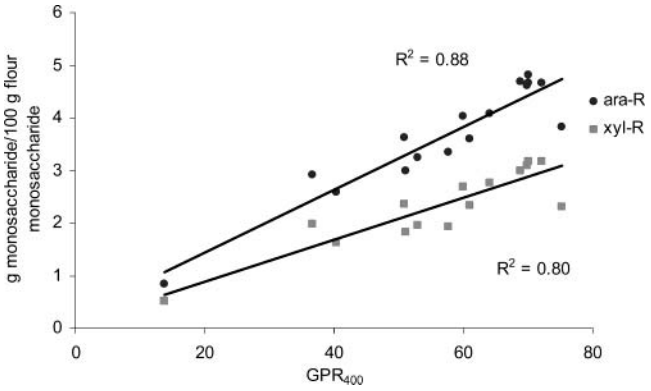

Figure 7. ara-R and xyl-R (percentage flour monosaccharide associated with the 400 μm gluten fraction: g monosaccharide/100 g flour monosaccharide) as a function of GPR on the 400 μm sieve (GPR_{400}).

et al. (*27*) also found an increasing amount of nonstarch polysaccharides associated with gluten as the GPR increased for six different wheat varieties.

DISCUSSION

Xylanase Addition at the Beginning of Mixing. The addition of xylanases to wheat flour changed the gluten agglomeration behavior during gluten−starch separation. The tested xylanases had a different functionality in the wheat flour separation process.

XAA improved gluten agglomeration and resulted in the formation of more large gluten aggregates. The GPAI increased from 83% for 0 U XAA to 93% for 0.14 U XAA/g flour. Increasing XAA dosages did not further improve the gluten agglomeration behavior. Further experiments have to be carried out to check whether similar or improved effects are observed when XAA is added to flours with moderate to bad processing properties. Although XAA also solubilized WU-AX, it mainly degraded WE-AX. The HPSEC profiles clearly indicate that the high molecular weight WE-AX (average molecular weight of 800×10^3) were degraded to lower molecular weight fragments. This degradation decreased viscosity of the dough and batter extracts.

Within the tested dosage range, XBS had a clearly negative effect on gluten agglomeration. With increasing XBS dosages, the GPR on the 400 μm sieve decreased and more gluten was retained on the 250 and 125 μm sieves. This can probably be explained by the increased viscosity of the batter by solubilization of high levels of WU-AX. It can be hypothesized that higher XBS dosages would further degrade S-AX and decrease batter viscosity, which would result in improved gluten agglomeration behavior.

When comparing the solubilization profiles as a function of XAA and XBS dosages (**Figure 3**), one should realize that the enzyme activities were determined with insoluble substrate (AZCL-AX) in the absence of WE-AX and inhibitors. Enzyme units determined with a soluble substrate would have been completely different because of the different substrate selectivity of XAA and XBS as indicated by their different substrate selectivity factors. Taking this in account, we would like to point out that from a scientific point of view, it is much more important to correlate xylanase functional effects with changes in the AX population and thus xylanase substrate selectivity than with xylanase activity measured on a single substrate.

The obtained results confirm that the viscosity of the batter is an important factor for the gluten agglomeration in the separation process. **Figure 8** shows the correlation between GPAI, a measure for the gluten agglomeration behavior, and the viscosity at the end of the batter phase. It indicates that a higher batter viscosity leads to a decreased GPAI. With addition of 1.60 U XBS/g flour, the viscosity and the GPAI do not follow this correlation. At this XBS dosage, an initial viscosity increase by solubilization of WU-AX was followed by degradation of high molecular weight S-AX and WE-AX, which decreased batter viscosity. This indicates that not the viscosity at the end of the process but the viscosity during an earlier phase of the batter process impacts gluten agglomeration. With addition of 1.60 U XBS/g flour, the GPAI indeed correlated better with dough extract viscosity (**Figure 8B**).

A tentative explanation for the correlation between GPAI and batter viscosity is the following. During the batter phase, the starch is washed out of the gluten matrix and the preformed (in dough mixing) gluten agglomerate. This process is negatively influenced by high batter viscosities, such as those resulting from the solubilization of WU-AX by the xylanase. The increased viscosity disturbs the interaction between gluten proteins and decreases the mobility of the components in the batter and the rate of gluten agglomeration. Degradation of WE-AX and S-AX decreases viscosity and increases availability of

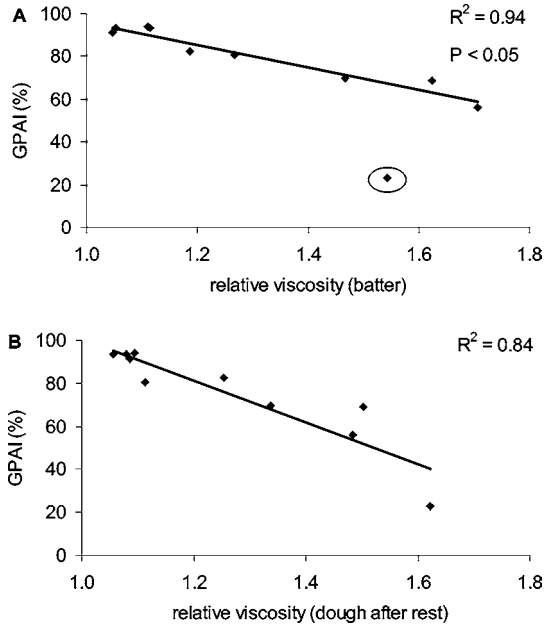

Figure 8. Correlation between GPAI (%) and relative viscosity of the batter extracts (**A**) and relative viscosity of the dough extracts (**B**) for xylanase additions in the beginning of the separation process. The encircled point is not included in the calculation of the correlation coefficient.

water for the gluten development and interaction. This is in agreement with the results of Frederix et al. (*11*) and Redgwell et al. (*12*) who also found that a batter viscosity decreasing xylanase has a positive impact on gluten agglomeration.

An alternative explanation is that high molecular weight AXs disturb the agglomeration of gluten proteins and that steric hindrance of these molecules is responsible for the negative effect on the interactions between gluten proteins. A similar hypothesis has been formulated by Roels et al. (*28*) in an attempt to explain the observation that the WE-AX content of wheat flour is negatively correlated with the Zeleny sedimentation value as a result of the disturbance of gluten protein flocculation by WE-AX. In the batter process, the beneficial effect of xylanases has been ascribed to a decreased interaction between gluten proteins and WE-AX (*12*) or removal of the steric hindrance that AXs exert on gluten agglomeration (*29*). Wang et al. (*2*) postulated that AX-bound ferulic acid can directly link to gluten proteins and in such a way negatively influence gluten formation.

The improved agglomeration as a result of the joint addition of high levels of XAA and XBS (5.6 U XAA and 16 U XBS/g flour) indicates that not only the viscosity caused by WE-AX and S-AX plays a determining role but also the presence of WU-AX. These WU-AX, which are present as cell wall fragments, can interfere with gluten agglomeration through steric hindrance and can form a physical barrier for the interaction between gluten particles. The action of the two enzymes together reduced the level of WU-AX with 80% and degraded S-AX to lower molecular weight fragments, which resulted in a low viscosity of the batter extract. In this context, a negative effect of added WU-AX on gluten formation was previously noted by Wang et al. (*3*). Also, the addition of wheat flour WU-AX to a commercial gluten−starch mixture influenced gluten agglomeration negatively during a batter procedure (*30*).

The above presented results indicate the importance of WE-AX as well as of WU-AX in gluten agglomeration behavior during gluten−starch separation. Therefore, it can be speculated that addition of very high dosages of XAA or XBS as any xylanase for that matter would probably improve gluten agglomeration to the same extent as with the combination of both enzymes because of the degradation of the complete AX population at these high xylanase dosages.

Because the two selected enzymes in this study have different substrate selectivities and inhibitor sensitivities, it is difficult to determine which factor is the most important for the difference in functionality of the two xylanases in gluten−starch separation. The drastic decrease (89%) in XBS activity (1.60 U/g flour) by inhibitors in Zohra wheat flour extract indicates that inhibition certainly plays a role in xylanase functionality. XBS was probably gradually inhibited as a function of process time. Further research will need to elucidate the exact role of substrate selectivity and inhibition in the process. Whatever the case is, we here speculate that a xylanase with optimum performance in gluten−starch separation is not inhibited and hydrolyzes both WE-AX and WU-AX to low molecular weight fragments.

Xylanase Addition at Different Stages of the Process. The addition of XAA and XBS to flour and to dough after resting resulted in the same gluten agglomeration behavior and approximately the same viscosity at the end of the batter phase. Here, a correlation between gluten agglomeration and viscosity of the batter exists.

The addition of xylanase 10 min before the end of the batter phase resulted in a similar distribution of gluten proteins over the sieves as in the control, despite the fact that the xylanase had influenced the AX population and the viscosity of the batter during these 10 min. This provides evidence that not the viscosity at the end of the process but that during the initial phase of the batter step is important for gluten agglomeration.

CONCLUSIONS

XAA decreased batter viscosity and the molecular weight of the WE-AX and S-AX, resulting in an improved gluten agglomeration behavior and the formation of more large gluten aggregates. In contrast, XBS solubilized high levels of WU-AX and thereby increased the viscosity of the batter. This had a negative effect on the gluten agglomeration. The GPR on the 400 μm sieve decreased, and more gluten was retained on the 250 and 125 μm sieve. At higher XBS dosages, the high molecular weight S-AXs were degraded, and the viscosity decreased. The two xylanases therefore clearly had a different functionality in gluten−starch separation. The results indicate that substrate selectivity and inhibitor sensitivity both are important factors for the performance of xylanases in this process and that further research is necessary to clarify their exact role.

The addition of XAA in combination with XBS showed a gluten distribution similar to that without xylanase addition. Apparently, XAA neutralized the effect of XBS. However, with high dosages of XAA and XBS together, the gluten agglomeration behavior improved to a larger extent than with the addition of XAA alone indicating the importance of both WE-AX and WU-AX in gluten−starch separation. Furthermore, these results suggest that the addition of very high XAA or XBS dosages would also improve gluten agglomeration drastically. Indeed, at very high xylanase dosages and whatever the xylanase substrate selectivity, most of the WU-AX will be solubilized and the S-AX will be degraded to lower molecular weight fragments resulting in low batter viscosities. Finally, the addition of xylanases at different moments during the separation process indicated that the viscosity during the initial phase of the batter step is important for the gluten agglomeration.

ABBREVIATIONS USED

ara-R, arabinose recovery; AX, arabinoxylan(s); AZCL-AX, azurine cross-linked arabinoxylan; GPAI, gluten protein agglomeration index; GPR, gluten protein recovery; GPR_{tot}, total gluten protein recovery; GPR_{xxx}, gluten protein recovery on the sieve with pore diameter of xxx μm; HPSEC, high-performance size exclusion chromatography; S-AX, solubilized arabinoxylan(s); TAXI, *Triticum aestivum* L. xylanase inhibitor(s); WE-AX, water extractable arabinoxylan(s); WU-AX, water unextractable arabinoxylan(s); XAA, xylanase from *Aspergillus aculeatus*; XBS, xylanase from *Bacillus subtilis*; XIP, xylanase inhibitor protein(s); xyl-R, xylose recovery.

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