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Impact of Inhibition Sensitivity on Endoxylanase Functionality in Wheat Flour Breadmaking

ISABEL TROGH,*,† JENS F. SØRENSEN,‡ CHRISTOPHE M. COURTIN,† AND JAN A. DELCOUR†

Laboratory of Food Chemistry, Katholieke Universiteit Leuven, Kasteelpark Arenberg 20, B-3001 Leuven, Belgium, and Danisco, Edwin Rahrs Vej 38, DK-8220 Brabrand, Denmark

A *Bacillus subtilis* endoxylanase (XBS_i) sensitive to inhibition by *Triticum aestivum* L. endoxylanase inhibitor (TAXI) and a mutant thereof (XBS_{ni}), uninhibited by TAXI, were used in straight-dough breadmaking to assess the importance of endoxylanase inhibition sensitivity on endoxylanase functionality in the process. With two European wheat flours, the loaf volume improving effect of XBS_{ni} at much lower enzyme dosages was substantially larger than that brought about by XBS_i. This coincided with differences in arabinoxylan (AX) hydrolysis. Although XBS_{ni} had a lower substrate selectivity for water-unextractable arabinoxylan (WU-AX) than XBS_i, the former solubilized significantly more WU-AX than XBS_i. Because of inhibition, XBS_i solubilized most of the WU-AX during mixing, whereas, with XBS_{ni}, the rate of solubilization decreased less with increasing processing time than that with XBS_i. During fermentation and baking and at the highest dosage (600 U/kg of flour of XBS_i and 60 U/kg of flour of XBS_{ni}), XBS_{ni} induced a stronger degradation of enzymically solubilized and water-extractable AX than XBS_i. Taken together, the data clearly demonstrate that endoxylanases, which in vitro are inhibited by endoxylanase inhibitors and still are active in the breadmaking process, as demonstrated by their functional (bread volume) enhancing effect, gradually lose their activity in the process.

KEYWORDS: Endoxylanase; inhibition; arabinoxylan; breadmaking

INTRODUCTION

Endo-β-1,4-xylanases (EC 3.2.1.8), hereafter referred to as endoxylanases, are routinely used in biotechnological processes such as breadmaking (*1*, *2*) and gluten−starch separation (*3*, *4*). They hydrolyze internal β-(1,4)-linkages in the backbone of arabinoxylan (AX), a nonstarch polysaccharide that constitutes the major component of wheat grain cell walls and that is either water-extractable (WE-AX) or water-unextractable (WU-AX). The latter AX have strong water-holding capacity, whereas the former form highly viscous aqueous solutions (*5*−*7*). WU-AX have a negative effect on bread characteristics, whereas WE-AX and solubilized AX (S-AX) are positive for breadmaking (*8*, *9*). Endoxylanases strongly affect AX structure and functionality. They can hydrolyze WU-AX, resulting in the release of S-AX and, consequently, in an increased viscosity of the aqueous phase. The viscosity decreases when S-AX and native WE-AX are degraded to low molecular weight (MW) AX fragments (*9*, *10*).

Endoxylanase functionality depends on several parameters such as the biochemical properties of the enzyme, substrate specificity (hydrolysis pattern) (*11*), and substrate selectivity, that is, their relative activity toward WE-AX and WU-AX (*12*, *13*). In agreement with the above, endoxylanases that preferentially hydrolyze WU-AX and leave WE-AX and S-AX unharmed are beneficial for breadmaking, whereas those that preferentially degrade WE-AX and S-AX have little effect (*2*, *9*).

Some seven years ago, proteinaceous inhibitors of endoxylanases were discovered in wheat (*14*). Two types of endoxylanase inhibitors with different structures and endoxylanase specificities have since been described, that is, *Triticum aestivum* L. endoxylanase inhibitor (TAXI)-like inhibitors (*15*−*17*) and endoxylanase inhibiting protein (XIP)-like inhibitors (*18*, *19*). Gebruers et al. (*20*) reported significant variation in the endoxylanase inhibitor activity (when assayed in vitro) in different European wheat cultivars and milling fractions. The presence of endogenous endoxylanase inhibitors can hence be expected to affect the functionality and performance of endoxylanases in biotechnological processes. Indeed, in general, the inhibitor levels in flours are higher than those of exogenous endoxylanases typically added (*21*). However, in the literature, very limited information (*15*, *22*) is available about the impact of endoxylanase inhibition (generally assayed in vitro with a flour water extract) on endoxylanase functionality in breadmaking.

* Corresponding author [telephone (+ 32) 16 321634; fax (+ 32) 16 321997; e-mail isabel.trogh@agr.kuleuven.ac.be].
† Katholieke Universiteit Leuven.
‡ Danisco.

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The purpose of this study was therefore to investigate the impact of inhibition by wheat endogenous endoxylanase inhibitors on the functionality of endoxylanases in wheat flour breadmaking, by using a *Bacillus subtilis* endoxylanase (currently used in many breadmaking systems) sensitive to inhibition and a mutant of the enzyme, which is no longer inhibited. Different dosages of the two enzymes were added, and their effects on dough and bread in general and on the AX population, in particular, was studied.

MATERIALS AND METHODS

Enzymes. A wild-type endoxylanase of *B. subtilis* (XBS_i) (SWISS-PROT entry P18429, XynA_BACSU) sensitive to inhibition by TAXI but not by XIP and a mutant thereof (XBS_{ni}) completely insensitive to inhibition by either TAXI or XIP were used (Danisco, Brabrand, Denmark). XBS_{ni} was obtained by site-directed mutagenesis and differed only in a few amino acids (D11F/R122D/Q175L) from XBS_i (*23*). Both endoxylanases were free from side activities and had similar pH and temperature stabilities. Their activity, determined on soluble AX (Megazyme, Bray, Ireland), was expressed in units, whereby 1 unit (U) is defined as the amount of endoxylanase preparation, diluted in sodium acetate buffer (100 mM, pH 5.0), that yields reducing-end groups equal to 1 μM xylose/min (*24*). The substrate selectivity factors of XBS_i and XBS_{ni} (representing the ratio of endoxylanase activity toward insoluble and soluble AX substrates), determined using the method of Moers et al. (*13*), were 22.0 and 6.4, respectively, indicating that XBS_i had a higher relative selectivity for WU-AX than XBS_{ni}.

α-Amylase (Grindamyl A1000, Danisco) was added in the breadmaking recipes, and the fungal α-amylase units (FAU) are used as specified by the supplier.

Flours. Two European wheat flours, both free from additives, were used for the breadmaking process. Reform flour was a commercial wheat flour from Havnemøllen (Cerealia, Sweden). Zohra wheat (AVEVE, Belgium) was conditioned to a moisture content of 14.5% and milled on a Bühler MLU-202 laboratory mill (Uzwill, Switzerland) into flour, bran, and shorts, according to AACC method 26-31 (*25*). Protein (% dm) and ash contents (% dm) were 13.0 and 10.4% and 0.77 and 0.57% for Reform and Zohra flours, respectively. The total AX contents (% dm) for Reform and Zohra flours were 2.11 and 2.05%, respectively.

Standard Analyses. Moisture and ash contents were estimated according to AACC methods 44-15A and 08-01, respectively (*25*). Protein content was determined using the Dumas method, an adaptation of AOAC method 990.03 (*26*) to an automated Dumas protein analysis system (EAS variomax N/CN, Elt, Gouda, The Netherlands).

Mixograph Measurement. For both flours, 10 g scale mixograms (National Manufacturing, Lincoln, NE) (AACC method 54-40A) (*25*) were recorded to determine the optimal mixing time.

Breadmaking Process. Doughs and breads were produced at a 100 g scale according to the straight-dough breadmaking procedure of Finney (*27*). Flour (100 g, 14% moisture base), fresh yeast (5.3%), salt (1.5%), sugar (6.0%), and α-amylase (75 FAU/kg of flour for Reform and 100 FAU/kg of flour for Zohra flour) were mixed with water (53.6% for Reform and 55.4% for Zohra flour) in a 100 g pin mixer (National Manufacturing) during 3.3 and 3 min for Reform and Zohra flours, respectively. For endoxylanase addition, the enzyme preparations were diluted in sodium acetate buffer (50 mM, pH 5.0). On the basis of preliminary breadmaking trials, dosages of 0, 24, 120, and 600 U/kg of flour were used for XBS_i and 0, 2.4, 12, and 60 U/kg of flour for XBS_{ni}. The first and second punches were after fermentation times of 52 and 77 min, respectively; the third punch and molding were after 90 min of fermentation. Finally, doughs were proofed (36 min) and baked at 215 °C for 24 min in a rotary oven (National Manufacturing). After 1 h at room temperature, the breads were weighed. Loaf volumes were measured by rapeseed displacement on three separate loaves.

Dough samples taken immediately after mixing, before the first punch and after proofing, and bread samples, withdrawn 24 h after baking, were frozen in liquid nitrogen. After lyophilization, they were ground and sieved (∅ = 250 μm).

Extraction of Flour, Dough, and Bread Samples. Aqueous extracts of flour, dough, fermented dough, and bread samples were prepared according to the method of Courtin et al. (*9*). Flour, dough, and bread samples (5.0 g) were extracted with deionized water (100 mL, 30 min, 4 °C). After centrifugation (10000*g*, 15 min, 4 °C), the supernatant was lyophilized and boiling water was added to the lyophilized material followed by a boiling step (30 min, 100 °C) to inactivate the enzymes. After lyophilization, the material was dispersed in water (50 mL) and centrifuged (10000*g*, 15 min, 4 °C), and the supernatant was frozen until further analysis.

Carbohydrate Composition. Carbohydrate compositions of flour, dough, and bread samples and aqueous extracts thereof were estimated by gas–liquid chromatography of alditol acetates. The flour and lyophilized samples (0.100 g) were hydrolyzed in 2.0 M trifluoroacetic acid (TFA) (5.0 mL) for 120 min at 110 °C and aqueous extracts (2.5 mL) in 4.0 M TFA (2.5 mL) for 60 min at 110 °C. Reduction was with sodium borohydride and acetylation with acetic anhydride (*28*). The formed alditol acetates (1.0 μL) were separated on a Supelco SP-2380 polar column (30 m × 0.32 mm i.d.; 0.2 μm film thickness) (Supelco, Bellefonte, PA) in an Agilent chromatograph (Agilent 6890 series, Wilmington, DE) equipped with autosampler, splitter injection port (split ratio 1:20), and flame ionization detector. The carrier gas was helium. Separation was at 225 °C, and injection and detection were at 270 °C. The coefficient of variation of the analysis was <5.0%. AX content was 0.88 times the sum of xylose and arabinose, after correction of the arabinose content for the presence of arabinogalactan-peptide based on an arabinose-to-galactose ratio of 0.7 and with the assumption that all of the arabinose of arabinogalactan-peptide is present in the aqueous extract (*29*).

High-Performance Size Exclusion Chromatography (HPSEC). The apparent molecular weight distribution of the components in the aqueous extracts of dough and bread samples was studied by HPSEC on a Shodex SB-806 HQ column (300 mm × 8 mm i.d.) with a Shodex SB-G guard column (50 mm × 6 mm i.d.) from Showa Denko K.K. (Tokyo, Japan). The separation range of the SB-806 HQ column was between 1 kDa and 20 MDa. Elution of the samples (20 μL) was with 0.3% NaCl (0.5 mL/min at 30 °C) on a Kontron 325 pump system (Kontron, Milan, Italy) with autoinjection. The separation was monitored with a refractive index (RI) detector (VDS Optilab, Berlin, Germany). MW markers (1.5 mg/mL) were Shodex standard P-82 pullulans from Showa Denko K.K. with MW 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.

Viscosity Measurement. The viscosity of the extracts of dough and bread samples was determined with an Ostwald-type viscometer at 30 °C according to the method of Vinkx et al. (*30*). Specific viscosity was defined as the relative viscosity (viscosity relative to that of deionized water) minus one.

RESULTS

Dough and Bread Characteristics. *Dough.* As too high dosages of endoxylanase can lead to a loss of dough water-holding capacity (*31*), preliminary breadmaking trials were performed to determine suitable dosages for both endoxylanases. For each combination of flour and endoxylanase, dough characteristics (elasticity, stickiness, and manageability) were assessed by an experienced baker at different stages during the process. On the basis of the results (not shown), dosages were fixed at 24, 120, and 600 U/kg of flour for XBS_i and at 2.4, 12, and 60 U/kg of flour for XBS_{ni}. At still higher dosages, the doughs had poor manageability. Within the set ranges and for both flours, the doughs made with XBS_i were elastic, well manageable, and not sticky. Only at 600 U/kg of flour of XBS_i, dough stickiness increased during the process. For XBS_{ni}, the highest dosage (60 U/kg of flour) resulted in doughs that were somewhat stickier than those with XBS_i. However, at dosages

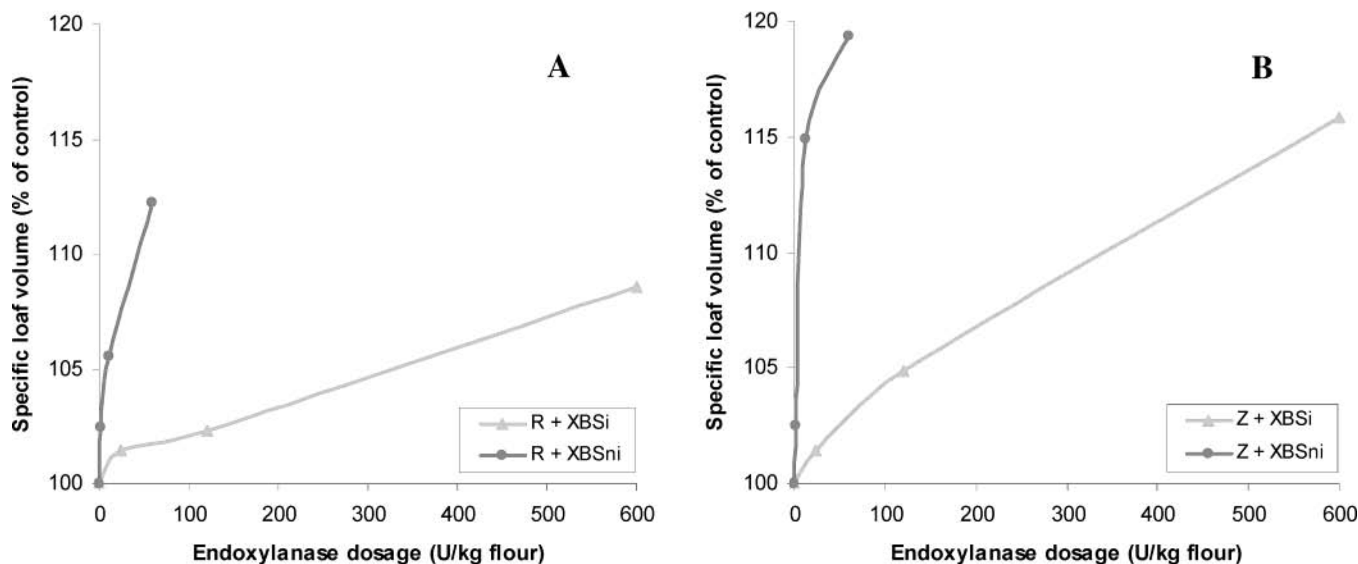

Figure 1. Specific loaf volumes (percent of control bread) of the breads made with Reform flour (R) (**A**) and Zohra flour (Z) (**B**) and different dosages of the inhibited endoxylanase of *B. subtilis* (XBS_i) and the uninhibited mutant (XBS_{ni}).

resulting in comparable volume increases in the final bread, no difference could be detected in dough stickiness.

Bread. The specific loaf volume of the control bread made with Reform flour (5.24 mL/g) was higher than that with Zohra flour (4.76 mL/g), in line with their relative protein contents. The specific loaf volume increases by XBS_i and XBS_{ni} increased with their dosages (**Figure 1**). At similar endoxylanase dosages, the loaf volume increase was much higher for the breads made with XBS_{ni} than for those made with XBS_i for both flours. Thus, at 60 U/kg of flour, the loaf volume increase could be estimated for Reform at 1.7 and 12.2% and for Zohra flour at 2.7 and 19.3% for XBS_i and XBS_{ni}, respectively. Even at the XBS_i highest dosage (600 U/kg of flour), the increase in loaf volume response was lower than that observed with XBS_{ni} (60 U/kg of flour) (**Figure 1**). The better response of Zohra flour was in line with the general observation that endoxylanases are more functional with weaker flours.

Both Reform and Zohra flour breads had good and homogeneous crumb structures and colors, irrespective of the endoxylanase dosage.

Solubilization of AX. Without enzyme addition, the breadmaking process led to only limited solubilization of WU-AX as observed earlier (*1*, *32*). **Figure 2** shows the solubilization of WU-AX during breadmaking with different dosages of XBS_i and XBS_{ni}.

At similar endoxylanase dosages (determined in vitro, i.e., in the absence of endoxylanase inhibitors) and at the same stage during breadmaking, solubilization of WU-AX was much higher for XBS_{ni} than for XBS_i, irrespective of the flour used. At 60 U/kg of flour, solubilization of WU-AX for Reform flour was 24 and 39% after mixing and 37 and 73% after proofing for XBS_i and XBS_{ni}, respectively. For Zohra flour, XBS_i and XBS_{ni} solubilized 18 and 40%, respectively, after mixing and 31 and 90%, respectively, after proofing. Thus, despite a lower substrate selectivity factor, XBS_{ni} (6.4) solubilized more WU-AX than XBS_i (22.0). This can be explained by the fact that XBS_{ni} is not inhibited by TAXI and constitutes direct proof for the impact of inhibitors on the functionality of endoxylanase in the breadmaking process.

Both endoxylanases solubilized relatively more WU-AX in Zohra than in Reform flour. Due to the higher ash content in Reform (0.77%) than in Zohra flour (0.57%), the former probably contained more bran WU-AX, which are much less liable to solubilization than endosperm WU-AX (*33*). For both flours, the maximum solubilization levels during fermentation and baking, obtained at 600 U/kg of flour for XBS_i and at 60 U/kg of flour for XBS_{ni}, were higher with XBS_{ni} than with XBS_i.

In general, XBS_i solubilized WU-AX mainly during mixing and less during fermentation, whereas XBS_{ni} solubilized most of the WU-AX during fermentation. The slower solubilization of WU-AX during fermentation by XBS_i than by XBS_{ni} may be explained by the fact that increasing levels of the XBS_i molecules were inhibited by TAXI as a function of time in the breadmaking process.

For both endoxylanases, the curve representing the levels of AX extractable after baking is positioned under those of AX extractable during the fermentation process. This indicates that part of the S-AX become unextractable again during baking due to physical inclusion or chemical linking of AX with themselves or with other bread components.

HPSEC Analysis and Molecular Weight of AX. Figure 3 shows the HPSEC molecular weight profiles of the aqueous extracts of dough, fermented dough, and bread samples made with Zohra flour and various dosages of XBS_i and XBS_{ni}. Similar profiles were obtained with Reform flour (profiles not shown).

During mixing (**Figure 3A**), increasing dosages of XBS_i and XBS_{ni} released high molecular weight (HMW) AX fragments (>200000). No significant degradation of WE-AX and S-AX was observed during mixing.

During fermentation (**Figure 3B**, **C**), the solubilization of WU-AX by XBS_i and XBS_{ni} was accompanied by the concomitant formation of AX fragments with a MW >100000. At the highest dosages of XBS_i (600 U/kg of flour) and XBS_{ni} (60 U/kg of flour), the RI response in the HMW region (>400000) decreased, indicating degradation of S-AX and/or WE-AX. At ~10-fold higher dosages of XBS_i, effects similar to those observed with XBS_{ni} could be seen.

The higher RI response of the curves of the control bread samples (**Figure 3D**) indicated an increase in the level of extractable material in the postfermentation stages of the breadmaking process. This is probably due to the formation of other water-extractable material during baking. At low XBS_i and XBS_{ni} dosages, WU-AX were solubilized, whereas at the highest

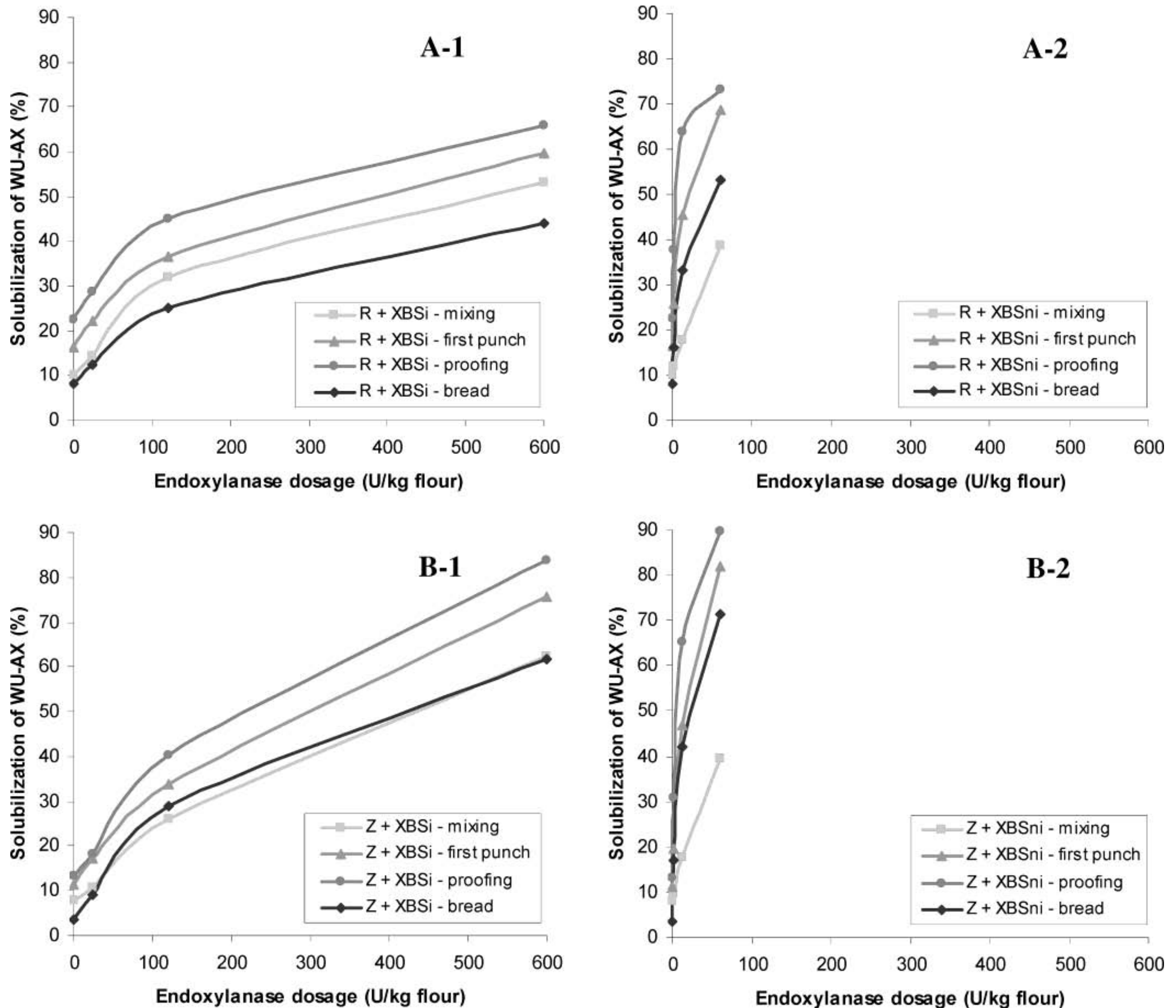

Figure 2. Solubilization (percent) of water-unextractable arabinoxylan (WU-AX) after mixing, before first punch, after proofing, and after baking for different dosages of the inhibited endoxylanase of *B. subtilis* (XBS_i) (**A-1**, **B-1**) and the uninhibited mutant (XBS_{ni}) (**A-2**, **B-2**) for Reform flour (R) (**A-1**, **A-2**) and Zohra flour (Z) (**B-1**, **B-2**).

enzyme dosage (600 U/kg of flour for XBS_i and 60 U/kg of flour for XBS_{ni}), AX degradation also was apparent. Again, an at least 10-fold higher dosage of XBS_i had to be added to observe this degradation effect.

Specific Viscosities of Dough and Bread Extracts. The specific viscosities of the aqueous extracts were measured for dough, fermented dough, and bread samples made with Reform and Zohra flours and different dosages of XBS_i and XBS_{ni} (**Figure 4**). For XBS_i, the specific viscosity increased during mixing and the first fermentation step. During further fermentation and baking, the specific viscosity increased at lower XBS_i dosages and decreased slowly at higher XBS_i dosages after reaching an optimum at 120 U/kg of flour for both flours. The solubilization of WU-AX increased viscosity, whereas the degradation of AX decreased it.

For XBS_{ni}, the solubilization of WU-AX increased specific viscosity during mixing. During fermentation and baking, the viscosity increased quickly at low enzyme dosages but dropped at higher enzyme levels (60 U/kg of flour), irrespective of the flour used. The solubilization of WU-AX was predominant over the degradation of AX at low enzyme dosages, resulting in an increase in specific viscosity, whereas the opposite was observed at higher enzyme dosages. The decrease in specific viscosity, caused by degradation of WE-AX and/or S-AX, was much stronger for XBS_{ni} than for XBS_i, again indicating that XBS_{ni} was more active than XBS_i because it is not inhibited.

DISCUSSION

At similar endoxylanase dosages and at the same stage during breadmaking, the solubilization of WU-AX was much higher for XBS_{ni} than for XBS_i, despite the fact that XBS_i had a higher selectivity for WU-AX than XBS_{ni}. The rate of solubilization of WU-AX by XBS_i slowed more rapidly than with XBS_{ni}. With the latter enzyme, the solubilization of WU-AX during fermentation was relatively more important. These findings, taken together with the fact that higher dosages of XBS_i were needed to obtain similar effects as those observed with XBS_{ni}, lead us to conclude that much of the functionality of XBS_i is lost by progressive inhibition of XBS_i by TAXI. XBS_{ni} provided higher

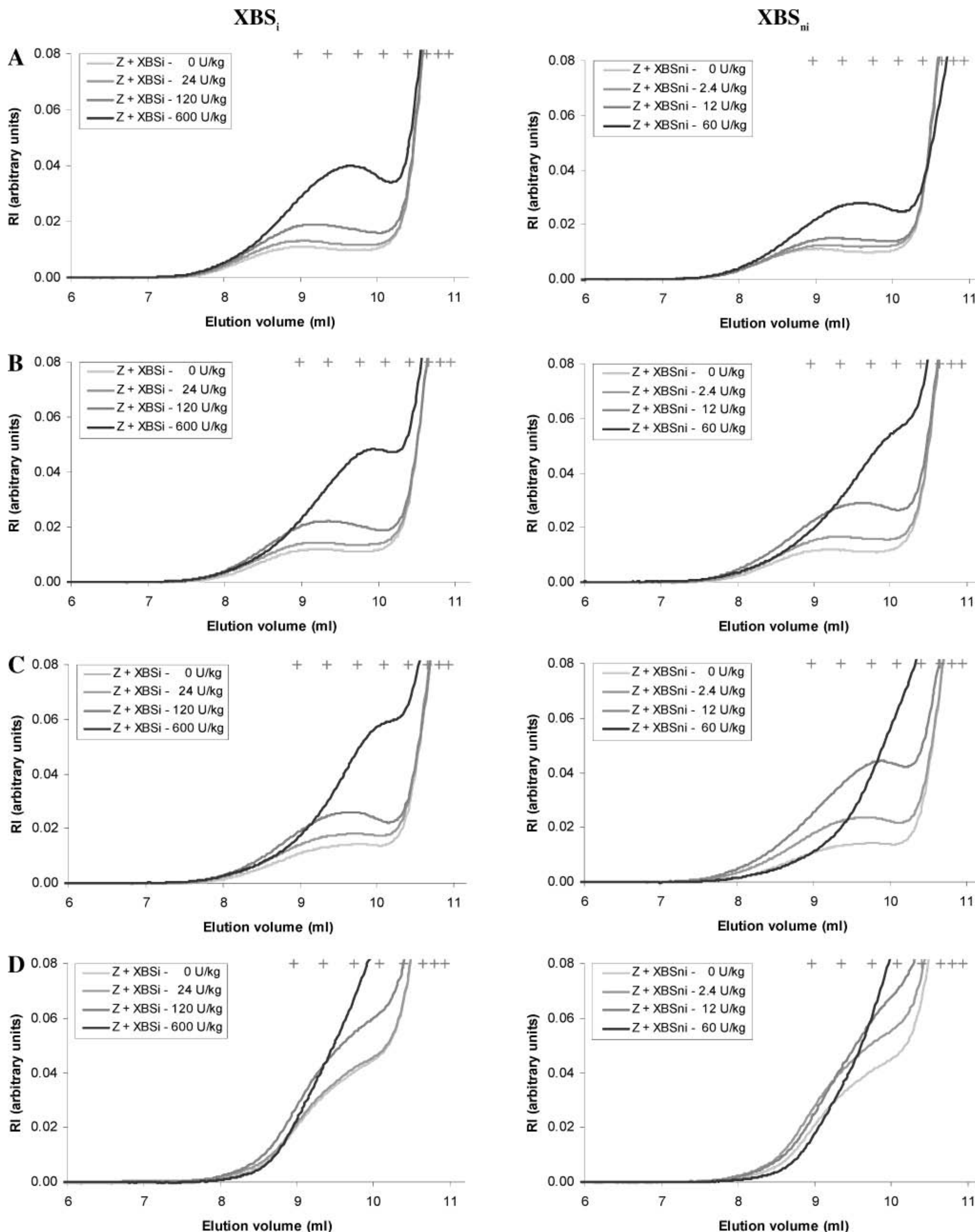

Figure 3. HPSEC molecular weight profiles of the aqueous extracts of dough after mixing (**A**), before first punch (**B**), after proofing (**C**), and after baking (**D**) for Zohra flour (Z) and different dosages (0–600 U/kg of flour) of the inhibited endoxylanase of *B. subtilis* (XBS_i) and the uninhibited mutant (XBS_{ni}). Molecular weight markers (+) from left to right 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, and 0.59×10^4.

WU-AX solubilization levels, also at later stages of breadmaking, and the formation of smaller AX fragments than XBS_i.

The extensive solubilization and degradation of AX by endoxylanases and hence also by XBS_i and XBS_{ni} during breadmaking and the resulting loss of water-holding capacity at high enzyme dosages induce dough stickiness. This was the limiting factor for the use of higher dosages of endoxylanases in our breadmaking process.

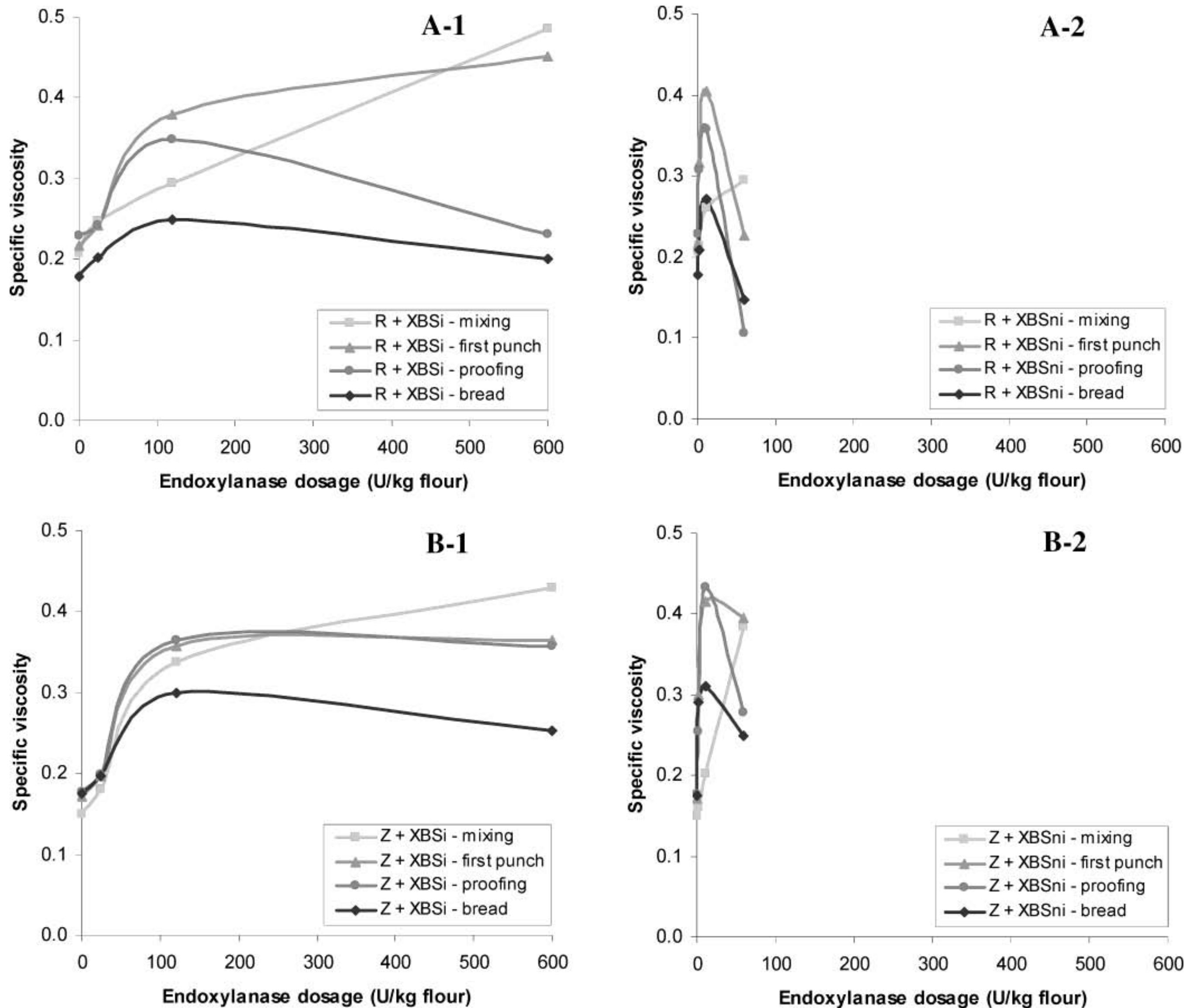

Figure 4. Specific viscosities of the aqueous extracts after mixing, before first punch, after proofing, and after baking for different dosages of the inhibited endoxylanase of *B. subtilis* (XBS_i) (**A-1**, **B-1**) and the uninhibited mutant (XBS_{ni}) (**A-2**, **B-2**) for Reform flour (R) (**A-1**, **A-2**) and Zohra flour (Z) (**B-1**, **B-2**).

For both flours, specific loaf volumes increased with increasing dosages of XBS_i and XBS_{ni}. At similar endoxylanase dosages, the increase in loaf volume was much higher for the breads made with XBS_{ni} than for those made with XBS_i. According to Courtin et al. (*8*, *9*), the increase in loaf volume is mainly due to the transformation of WU-AX into S-AX. The present observation can accordingly be explained by the higher solubilization levels of WU-AX obtained with XBS_{ni} than with XBS_i, due to the much higher effective activity of the uninhibited XBS_{ni}.

Because XBS_{ni} is not inhibited by either TAXI or XIP, it is more active on the AX population during breadmaking than XBS_i. This implies that much lower dosages of XBS_{ni} can be used when compared to those of XBS_i. Another advantage of the inhibition insensitivity of XBS_{ni} is a more predictable response when using this endoxylanase on flours with different inhibitor levels in cereal-based processes.

CONCLUSIONS

The present results clearly show the importance of endoxylanase inhibition sensitivity on endoxylanase functionality in breadmaking. XBS_i lost most of its functionality during fermentation. For both flours, higher specific loaf volumes were obtained with XBS_{ni} than with XBS_i, probably because more WU-AX were solubilized with XBS_{ni} than with XBS_i. During fermentation and baking and at the highest used dosage of XBS_{ni}, solubilization of WU-AX was really high and degradation of AX into smaller fragments took place. The combination of both actions resulted in sticky doughs during fermentation. On a unit basis, XBS_{ni} was at least 10 times more active on AX in breadmaking because it is insensitive to inhibition by TAXI.

The functionality and performance of endoxylanases in biotechnological processes hence is affected not only by the biochemical properties, substrate specificity, and substrate selectivity of the enzyme but also by its inhibition sensitivity.

ABBREVIATIONS USED

AX, arabinoxylan; dm, dry matter; FAU, fungal α-amylase unit; HMW, high molecular weight; HPSEC, high-performance size exclusion chromatography; MW, molecular weight; RI, refractive index; S-AX, solubilized arabinoxylan; TAXI, *Triticum aestivum* L. endoxylanase inhibitor; TFA, trifluoroacetic acid; U, units; WE-AX, water-extractable arabinoxylan; WU-

AX, water-unextractable arabinoxylan; XBS_i, inhibited wild-type endoxylanase of *Bacillus subtilis*; XBS_{ni}, not-inhibited mutant of the wild-type *B. subtilis* endoxylanase; XIP, endoxylanase inhibiting protein.

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