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Carbohydrate Research 340 (2005) 1319–1327
Carbohydrate RESEARCH

# Endoxylanase substrate selectivity determines degradation of wheat water-extractable and water-unextractable arabinoxylan

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Received 19 January 2005; received in revised form 25 February 2005; accepted 28 February 2005
Available online 9 April 2005

**Abstract**—The relative activity of an endoxylanase towards water-unextractable (WU-AX) and water-extractable arabinoxylan (WE-AX) substrates, referred to as endoxylanase substrate selectivity, impacts the enzyme functionality in cereal-based biotechnological processes such as bread-making and gluten starch separation. A set of six endoxylanases representing a range of substrate selectivities as determined by a screening method using chromophoric substrates [*Anal. Biochem.* **2003**, *319*, 73–77] was used to examine the impact of such selectivity on changes in structural characteristics of wheat WU-AX and WE-AX upon enzymic hydrolysis. While WE-AX degradation by the selected endoxylanases was very comparable with respect to apparent molecular mass (MM) profiles and arabinose to xylose ratio of the hydrolysates formed, WU-AX solubilisation and subsequent degradation of solubilised fragments gave rise to widely varying MM profiles, depending on the substrate selectivity of the enzymes. Enzymes with high selectivity towards WU-AX de facto generated higher MM fragments from WU-AX than enzymes with low selectivity. The arabinose to xylose ratios of solubilised fragments were independent of the degree of solubilisation.


*Keywords:* Endoxylanase; Substrate selectivity; Water-extractable arabinoxylan; Water-unextractable arabinoxylan

## 1. Introduction

Arabinoxylans (AX) are nonstarch polysaccharides of cereal grain cell wall material consisting of a backbone of xylose moieties substituted with arabinose residues.[1,2] AX can be divided into water-extractable (WE-AX) and water-unextractable AX (WU-AX), which have different physicochemical properties.[3] While WU-AX are retained in the cell wall by covalent and noncovalent interactions with other AX molecules and cell wall constituents such as protein, lignin, cellulose or β-glucan, WE-AX are thought to be loosely bound at the surface of these cell walls.[4,5] In biotechnological applications, (1→4)-β-D-xylan xylanohydrolases (EC 3.2.1.8; further referred to as endoxylanases), enzymes that hydrolyse AX, are frequently used to modify the functionality of AX. They attack the AX backbone in a random manner, solubilise WU-AX and degrade WE-AX and solubilised AX (S-AX) to lower molecular masses (MM).[6,7] Endoxylanases that differ in preference for both AX populations and, hence, their substrate selectivity, affect AX in different applications. Thus, a glycoside hydrolase family (GHF) 11 *Bacillus subtilis* endoxylanase, which preferentially hydrolyses WU-AX and leaves WE-AX and S-AX unharmed, beneficially affects bread loaf volume,[8,9] while in industrial wheat gluten starch separation, a GHF 10 endoxylanase of *Aspergillus aculeatus*, which preferentially degrades WE-AX and S-AX, improves gluten agglomeration.[10,11]

*Abbreviations*: AX, arabinoxylan(s); A/X, arabinose to xylose ratio; DP, average degree of polymerisation; GHF, glycoside hydrolase family; GLC, gas liquid chromatography; HPSEC, high-performance size exclusion chromatography; MM, molecular mass(s); RX, reducing xylose; S-AX, solubilised arabinoxylan(s); $SSF_{CHROM}$, substrate selectivity factor(s) determined using chromogenic substrates; $SSF_{NAT}$, substrate selectivity factor(s) determined using native substrates; SX, solubilised xylose; WE-AX, water-extractable AX; WU-AX, water-unextractable AX

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doi:10.1016/j.carres.2005.02.031

In a recent screening method for the evaluation of endoxylanase substrate selectivity using microtitre plates, a substrate selectivity factor based on chromophoric substrates ($SSF_{CHROM}$) was defined as the ratio of solubilising activity towards insoluble chromophoric AX substrate (based on solubilisation) over enzyme activity towards soluble substrates.[12] Endoxylanases analysed with this screening method showed a range of $SSF_{CHROM}$ values. However, the strong and at the same time weak point of this screening method is that it characterises an endoxylanase with a single number. On the one hand, this allows the easy comparison of enzymes, but on the other hand it does not provide detailed information on the hydrolysis of natural AX substrates. In addition, it has not been established whether the degradation of chromogenic and hence artificial substrates by different endoxylanases correlates with the degradation of their natural counterparts. We evaluate here the relationship between both aspects. To this end, six industrially relevant pure endoxylanases, differing in substrate selectivity as determined by the screening method, were selected. The degradation of native WU-AX and WE-AX substrates by these endoxylanases was analysed and the apparent MM and arabinose to xylose ratios (A/X) of the resulting AX fragments were determined. The results were related to the substrate selectivity values previously determined. We report here the outcome of this work.

## 2. Results and discussion

### 2.1. Enzyme activities on WU-AX and WE-AX

**2.1.1. Solubilisation of WU-AX.** The solubilisation of WU-AX was evaluated by measurement of (mostly polymeric) solubilised xylose (SX) released into the supernatant after incubation of WU-AX with different endoxylanase dosages. At low-enzyme dosages, WU-AX solubilisation was linearly related with enzyme concentration for all endoxylanases, allowing us to define the 'solubilising activity' ($A_{SX,WU\text{-}AX}$) (see Experimental). Table 1 presents specific solubilising activities ($A_{SX,WU\text{-}AX}$/mg) for all tested endoxylanases. The *B. subtilis* endoxylanase had the highest specific solubilising activity ($A_{SX,WU\text{-}AX}$/mg) and formed more SX at similar enzyme dosages than the *A. aculeatus* endoxylanase, with the lowest specific solubilising activity ($A_{SX,WU\text{-}AX}$/mg). A gradual decrease in specific solubilising activities ($A_{SX,WU\text{-}AX}$/mg) was observed ranging from *B. subtilis*, *Trichoderma longibrachiatum* (pI 5.5 enzyme), *Aspergillus niger*, *T. longibrachiatum* (pI 9.0 enzyme), *Trichoderma viride* to *A. aculeatus* endoxylanases.

A maximum of 70–80% solubilisation was reached at higher enzyme dosages, in agreement with earlier reports that enzymes do not completely solubilise WU-AX.[3,13] The A/X ratio of the solubilised fragments were ca. 0.46, irrespective of the degree of solubilisation (results not shown).

**2.1.2. Formation of carbohydrate reducing ends.** Enzyme activity towards WE-AX or WU-AX, based on the formation of reducing xylose (RX) was evaluated by measurement of RX released in the supernatant after incubation of WE-AX or WU-AX with different endoxylanase dosages. A linear relationship between RX formation and endoxylanase dosage was found when incubating both WE-AX and WU-AX, allowing the definition of 'RX forming activity' towards WE-AX ($A_{RX,WE\text{-}AX}$) as well as WU-AX ($A_{RX,WU\text{-}AX}$) (see Experimental). For all endoxylanases, solubilisation of WU-AX occurs fast, even at low-enzyme dosages. As a consequence, soluble AX is present in all incubation experiments with WU-AX. This implies that, when measuring the RX forming activity on WU-AX, there are actually two substrates present (WU-AX and S-AX), and a combination of RX forming activity on S-AX and WU-AX is measured. Specific enzyme activity towards WU-AX ($A_{RX,WU\text{-}AX}$) should therefore be

**Table 1.** Activities of six endoxylanases versus native wheat AX substrates: specific solubilising activity upon incubation of WU-AX with endoxylanase ($A_{SX,WU\text{-}AX}$/mg), specific enzyme activity towards WE-AX ($A_{RX,WE\text{-}AX}$/mg) and towards WU-AX ($A_{RX,WU\text{-}AX}$/mg) based on the formation of reducing xylose (RX), average degree of polymerisation of the xylan backbone of the solubilised AX fragments ($DP_{S\text{-}AX}$) and substrate selectivity factor determined with native AX ($SSF_{NAT}$) as well as chromophoric substrates ($SSF_{CHROM}$) are represented

| Endoxylanase source | $A_{SX,WU\text{-}AX}$/mg (nKat/mg) | $A_{RX,WE\text{-}AX}$/mg (nKat/mg) | $A_{RX,WU\text{-}AX}$/mg (nKat/mg) | $SSF_{NAT}$[a] | $SSF_{CHROM}$[b] | $DP_{S\text{-}AX}$[c] |
|---|---|---|---|---|---|---|
| *B. subtilis* | 451,030 | 1230 | 1075 | 366 | 22.0 | 420 |
| *A. niger* | 205,700 | 1480 | 1385 | 139 | 7.9 | 150 |
| *T. longibrachiatum*[d] | 67,690 | 650 | 630 | 104 | 6.4 | 100 |
| *T. longibrachiatum*[e] | 62,620 | 2000 | 1770 | 31 | 3.4 | 35 |
| *T. viride* | 9530 | 270 | 310 | 36 | 2.7 | 30 |
| *A. aculeatus* | 9420 | 785 | 620 | 12 | 0.9 | 15 |

[a] $SSF_{NAT} = \frac{A_{SX,WU\text{-}AX}/\text{mg}}{A_{RX,WE\text{-}AX}/\text{mg}}$.

[b] Determined in Moers et al.[12]

[c] $DP_{S\text{-}AX} = \frac{A_{SX,WU\text{-}AX}/\text{mg}}{A_{RX,WU\text{-}AX}/\text{mg}}$.

[d] Enzyme with pI 9.0.

[e] Enzyme with pI 5.5.

considered as an apparent activity. This probably explains the strong resemblance in specific enzyme activity towards WU-AX ($A_{RX,WU\text{-}AX}$/mg) and that towards WE-AX ($A_{RX,WE\text{-}AX}$/mg) for all enzymes (Table 1). Furthermore, specific solubilising activities ($A_{SX,WU\text{-}AX}$/mg) were not linearly related to specific enzyme activities towards WU-AX ($A_{RX,WU\text{-}AX}$/mg), indicating differences in MM of the formed fragments as reflected in the degree of polymerisation (DP) (cfr. infra.).

## 2.2. Apparent MM of AX fragments

Incubation of WE-AX with increasing dosages of endoxylanases showed a gradual shift in apparent MM of the formed AX fragments to lower MM (Fig. 1). For all endoxylanases, the profiles of the AX fragments upon incubation of WE-AX with endoxylanases were very similar.

Incubation of WU-AX with low dosages of endoxylanases led to solubilisation and appearance of S-AX fragments in HPSEC profiles (Fig. 2). At higher enzyme dosages, more solubilisation occurred and S-AX fragments were degraded to lower MM. The extent of WU-AX solubilisation on the one hand, and the degradation of the formed S-AX fragments on the other hand, depend on $SSF_{CHROM}$ of the endoxylanases. The *B. subtilis* endoxylanase at low-enzyme dosages showed S-AX fragments of high MM in the HPSEC profiles: at an

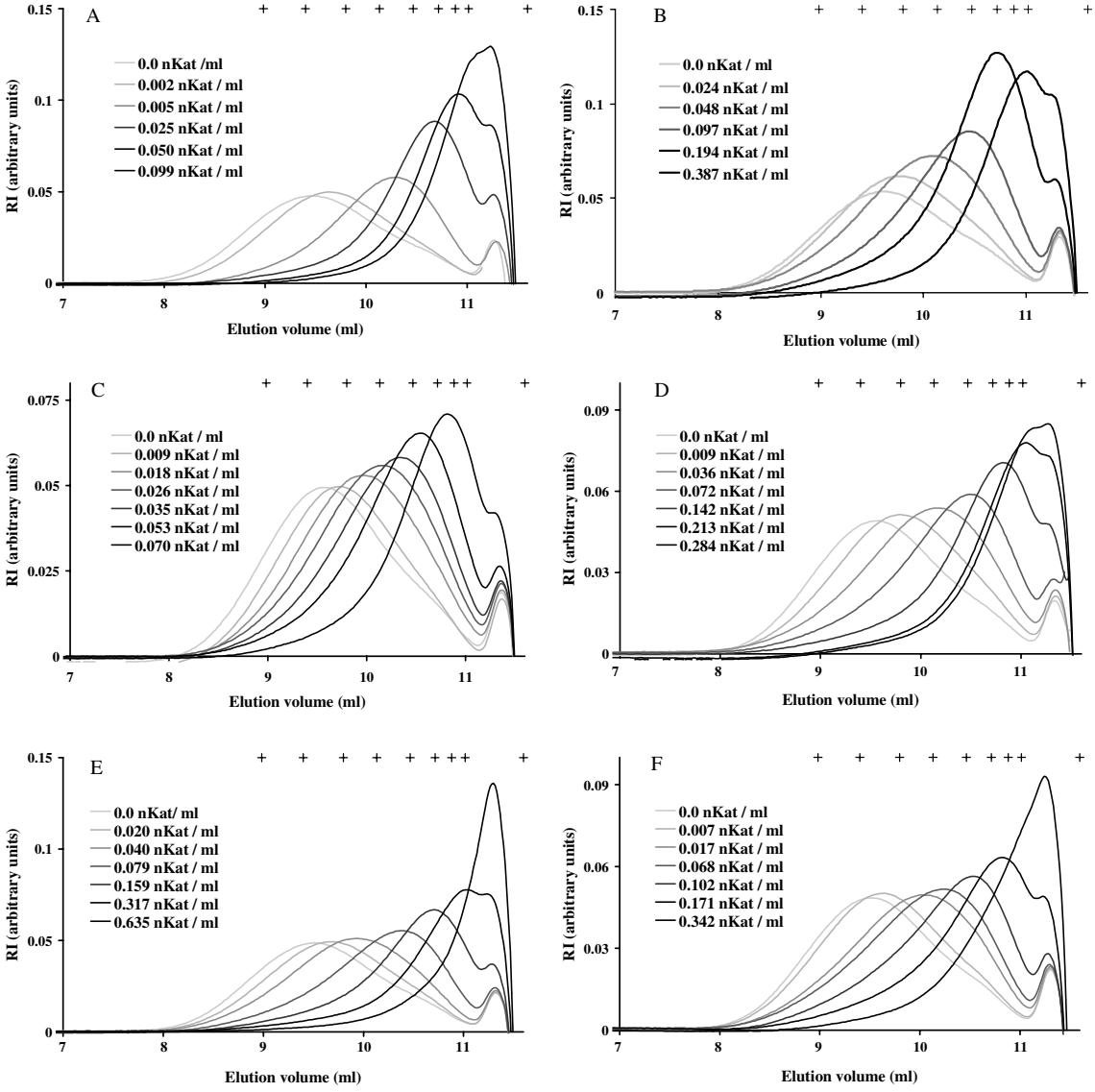


**Figure 1.** HPSEC profiles of AX fragments obtained by incubation of WE-AX with different dosages of endoxylanases from *B. subtilis* (A), *A. niger* (B), *T. longibrachiatum* (C, pI 5.5), *T. longibrachiatum* (D, pI 9.0), *T. viride* (E) and *A. aculeatus* (F). The HPSEC profiles are represented in order of decreasing substrate selectivity factor ($SSF_{CHROM}$) of the endoxylanases. Elution times of pullulan standards of MM of $78.8 \times 10^4$, $40.4 \times 10^4$, $21.2 \times 10^4$, $11.2 \times 10^4$, $4.73 \times 10^4$, $2.28 \times 10^4$, $1.18 \times 10^4$ and $0.59 \times 10^4$ Da and glucose are indicated from left to right (+). Enzyme dosages (nKat/mL) are expressed as enzyme activity towards WE-AX ($A_{RX,WE\text{-}AX}$).

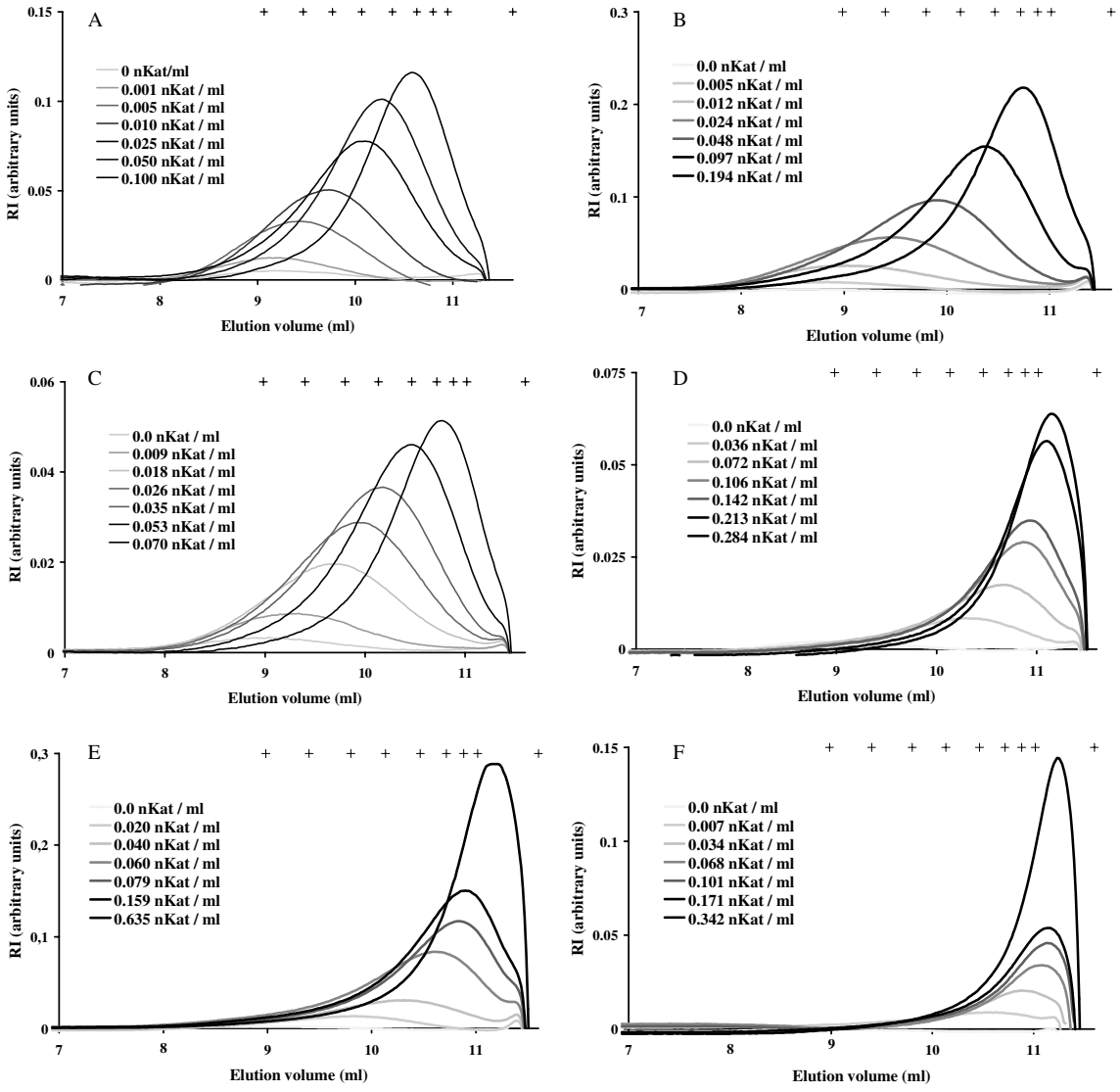


**Figure 2.** HPSEC profiles of S-AX fragments obtained by incubation of WU-AX with different dosages of endoxylanases from *B. subtilis* (A), *A. niger* (B), *T. longibrachiatum* (C, pI 5.5), *T. longibrachiatum* (D, pI 9.0), *T. viride* (E) and *A. aculeatus* (F). The HPSEC profiles are represented in order of decreasing substrate selectivity factor ($SSF_{CHROM}$) of the endoxylanases. Elution times of pullulan standards of MM of $78.8 \times 10^4$, $40.4 \times 10^4$, $21.2 \times 10^4$, $11.2 \times 10^4$, $4.73 \times 10^4$, $2.28 \times 10^4$, $1.18 \times 10^4$ and $0.59 \times 10^4$ Da and glucose are indicated from left to right (+). Enzyme dosages (nKat/mL) are expressed as enzyme activity towards WE-AX ($A_{RX,WE\text{-}AX}$).

endoxylanase dosage of 0.005 nKat/mL ($A_{RX,WE\text{-}AX}$/mL; 58% xylose solubilisation), the apparent peak MM was around 410 kDa (Fig. 2A). At higher enzyme dosages, AX fragments with lower MM were found because of S-AX degradation. On the contrary, S-AX profiles for the *A. aculeatus* endoxylanase showed fragments of low apparent MM already at low-enzyme dosages (Fig. 2F). At a dosage of 0.034 nKat/mL ($A_{RX,WE\text{-}AX}$/mL) *A. aculeatus* endoxylanase, the apparent peak MM was around 12 kDa while only 13% of all xylose of the original WU-AX material was solubilised. No high MM S-AX fragments were observed in the HPSEC profiles of S-AX fragments after incubation of WU-AX with the *A. aculeatus* endoxylanase. Probably, degradation of these S-AX fragments occurred at a much higher rate than solubilisation. The profiles of the S-AX fragments obtained by incubation of WU-AX with different dosages of *T. longibrachiatum* (pI 5.5) and *A. niger* endoxylanases were comparable to those obtained with the *B. subtilis* endoxylanase, but, at a similar degree of solubilisation, these two endoxylanases degraded S-AX to lower MM than the *B. subtilis* endoxylanase. The profiles obtained with *T. longibrachiatum* (pI 9.0) and *T. vi-ride* endoxylanases resembled those of the S-AX fragments from WU-AX generated with the *A. aculeatus* endoxylanase, but degradation was not as extensive. S-AX formed after incubation of WU-AX with the *T. longibrachiatum* (pI 9.0) and the *T. viride* endoxylanases (Fig. 2) had higher MM than those produced by the *A. aculeatus* endoxylanase.

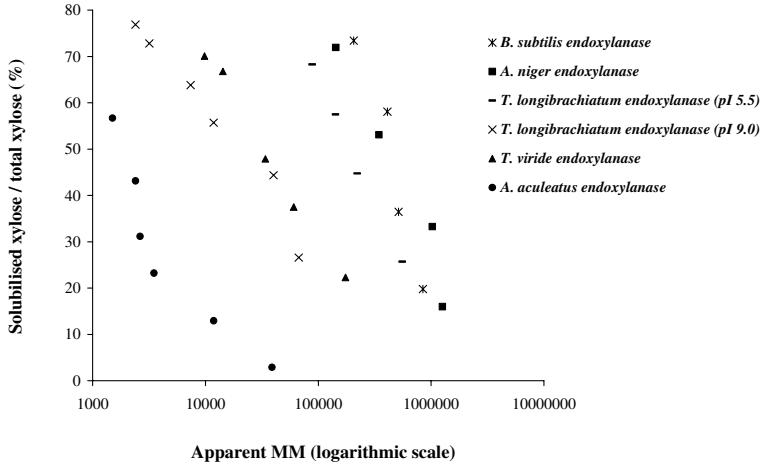


**Figure 3.** Correlation between the solubilised xylose (expressed as percentage of the total xylose content (%) in the original WU-AX material) and the apparent MM (logarithmic scale) of the formed AX fragments upon incubation of WU-AX with endoxylanases from *B. subtilis*, *A. niger*, *T. longibrachiatum* (pI 5.5), *T. longibrachiatum* (pI 9.0), *T. viride* and *A. aculeatus*.

In Figure 3, the percentage of SX is plotted against the apparent MM (logarithmic scale) of the resulting S-AX fragments that are produced upon incubation of WU-AX. Based on Figures 2 and 3, two main conclusions can be drawn. On the one hand, an increase in solubilisation is correlated with a decrease in S-AX fragment MM for all endoxylanases. On the other hand, for each endoxylanase it is clear that, at a certain solubilisation percentage, the MM is determined by its $SSF_{CHROM}$. For the *A. aculeatus* endoxylanase, only S-AX fragments of relatively low MM were formed even at low-solubilising activities. The *T. longibrachiatum* (pI 9.0) and *T. viride* endoxylanases produced S-AX fragments with higher MM. The endoxylanases from *T. longibrachiatum* (pI 5.5), *A. niger* and *B. subtilis* produced AX fragments with much higher MM even at high degrees of solubilisation.

### 2.3. Substrate selectivity

In the screening method for determination of endoxylanase substrate selectivity described earlier,[12] solubilising activity towards WU-AX $[(\Delta Y/\Delta X)_I]$ was estimated colourimetrically in microtitre plates using insoluble azurine crosslinked wheat AX as substrate. A similar approach using soluble azo wheat AX as substrate and ethanol precipitation of soluble undegraded AX fragments, was used to estimate endoxylanase activity towards WE-AX $[(\Delta Y/\Delta X)_S]$. A factor determining the substrate selectivity ($SSF_{CHROM}$) was defined as the ratio of both activities:

$$SSF_{CHROM} = \frac{(\Delta Y/\Delta X)_I}{(\Delta Y/\Delta X)_S} \quad (1)$$

A high $SSF_{CHROM}$ indicates that the endoxylanase preferentially hydrolyses WU-AX over WE-AX, while an endoxylanase with a lower $SSF_{CHROM}$ more readily hydrolyses WE-AX. By analogy with $SSF_{CHROM}$, and based on the activities presented in Table 1, a substrate selectivity factor determined with native wheat AX substrates ($SSF_{NAT}$) was defined as:

$$SSF_{NAT} = \frac{A_{SX,WU\text{-}AX}/mg}{A_{RX,WE\text{-}AX}/mg} \quad (2)$$

A high $SSF_{NAT}$ was found for the *B. subtilis* endoxylanase (366; see Table 1). The *A. aculeatus* endoxylanase on the contrary, had a low $SSF_{NAT}$ (12). $SSF_{NAT}$ for the four other endoxylanases varied in between these extremes in this order: *A. niger*, *T. longibrachiatum* (pI 5.5 enzyme), *T. viride* and *T. longibrachiatum* (pI 9.0 enzyme) endoxylanases.

The strong linear relation between $SSF_{NAT}$ and $SSF_{CHROM}$ for the six endoxylanases tested ($R^2 = 0.99$) (Fig. 4) indicates good agreement between substrate selectivities measured on chromophoric and native AX

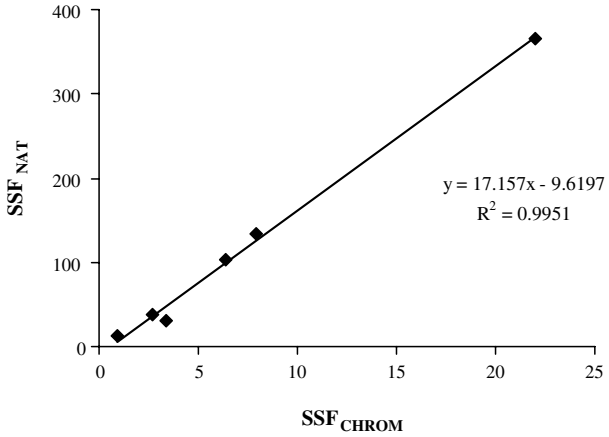


**Figure 4.** Relationship between the substrate selectivity factor based on native AX substrates ($SSF_{NAT}$) determined with the incubation experiments and the substrate selectivity factor based on chromophoric substrates ($SSF_{CHROM}$) determined with the screening method.

substrates. This was surprising as the methods for determination of endoxylanase activity towards soluble chromophoric AX substrate on the one hand, and wheat WE-AX on the other hand, differ. In the screening method, endoxylanase activity was measured based on changes in ethanol precipitation of chromophoric soluble AX before and after incubation with the enzyme, while in the incubation experiments the formation of RX from WE-AX was determined by gas liquid chromatography (GLC).

The average degree of polymerisation of the xylan backbone of the S-AX fragments ($DP_{S\text{-}AX}$) recovered after incubation of WU-AX with endoxylanases, was calculated as the ratio of specific solubilising activity ($A_{SX,WU\text{-}AX}$/mg) and specific enzyme activity towards WU-AX ($A_{RX,WU\text{-}AX}$/mg):

$$DP_{S\text{-}AX} = \frac{A_{SX,WU\text{-}AX}/\text{mg}}{A_{RX,WU\text{-}AX}/\text{mg}} \quad (3)$$

$DP_{S\text{-}AX}$ values varied in the same order as $SSF_{NAT}$ values. Indeed, a high $DP_{S\text{-}AX}$ resulted from high levels of solubilisation of WU-AX and a low formation of reducing ends. The *B. subtilis* endoxylanase solubilised much WU-AX during incubation while S-AX fragments were only slowly degraded to lower MM, as can be concluded from their high degree of polymerisation ($DP_{S\text{-}AX}$ = 420). In contrast, the *A. aculeatus* endoxylanase solubilised low levels of WU-AX, but the resulting S-AX were degraded rapidly to low MM ($DP_{S\text{-}AX}$ = 15). $DP_{S\text{-}AX}$ values of the other endoxylanases were intermediate. For the *A. niger* and the *T. longibrachiatum* (pI 5.5) endoxylanase products, these values were comparable (150 and 100, respectively), as were those for the *T. longibrachiatum* (pI 9.0) and *T. viride* endoxylanase products (35 and 30, respectively). Endoxylanases with a relatively high $SSF_{NAT}$ and $DP_{S\text{-}AX}$ [such as those from *B. subtilis*, *A. niger*, *T. longibrachiatum* (pI 5.5)] preferentially solubilised WU-AX, while those of *T. longibrachiatum* (pI 9.0), *T. viride* and *A. aculeatus* endoxylanases, with much lower $SSF_{NAT}$ and $DP_{S\text{-}AX}$ values, more readily hydrolysed WE-AX.

In this study, the impact of endoxylanase substrate selectivity on changes in structural characteristics and MM of wheat WU-AX and WE-AX upon enzymic hydrolysis was tested for six endoxylanases. A gradual decrease in specific solubilising activity of the endoxylanases was observed in accordance with their substrate selectivity factors determined earlier ($SSF_{CHROM}$). Furthermore, WU-AX solubilisation and subsequent degradation of S-AX fragments by the selected endoxylanases gave rise to widely differing apparent MM profiles, depending on the substrate selectivity of the enzymes. Enzymes with selectivity towards WU-AX generated higher MM fragments from WU-AX compared to enzymes with low such selectivity. A/X ratios of solubilised fragments were independent of the degree of solubilisation. The MM of the AX fragments formed upon incubation of WE-AX with different levels of each endoxylanase were very similar.

By analogy with the substrate selectivity factor determined with chromogenic substrates ($SSF_{CHROM}$), a substrate selectivity factor was determined with native wheat AX substrates ($SSF_{NAT}$). A strong linear relation between $SSF_{NAT}$ and $SSF_{CHROM}$ was found for the six endoxylanases tested. Endoxylanase SSF, determined both with native wheat AX substrates and with chromogenic AX substrates in the screening method, provides a valuable prediction of endoxylanase functionality in biotechnological processes.

In spite of reports in literature on different substrate specificities and degradation patterns for different endoxylanases and their relation to enzyme efficiency, the integrated concept of substrate selectivity is rather new. Its biochemical basis remains as yet unknown. It is tempting to attribute differences in substrate selectivity to differences in substrate specificity. It is indeed frequently reported that endoxylanases can give rise to different hydrolysis products and have different substrate specificity.[13–16] This is mainly associated with their classification in different GHF. However, the present study shows that endoxylanases within the same GHF and thus with similar specificity differ substantially in substrate selectivity. Related to this, the A/X ratio of the substrates could be a factor. However, because the average A/X ratios of WE-AX and WU-AX used in this study are very similar and only small differences have been reported in WE-AX and WU-AX structure, this will probably not contribute to differences in substrate selectivity. As with amylases,[17] different enzyme attack mechanisms could be in place, but also here the present data does not seem to support this hypothesis. Further research will be necessary to understand the mechanisms underlying endoxylanase substrate selectivity. It is clear that, until that time, substrate selectivity should be regarded as functional and practical concept rather than a biochemical one.

## 3. Experimental

### 3.1. Materials

Wheat (cv. Petrus from Clovis Matton, Avelgem, Belgium) was conditioned to 16.0% moisture and milled with a Bühler MLU-202 Laboratory Mill (Uzwil, Switzerland) according to AACC Method 26–31.[18] Moisture (% dm, AACC Method 44-15A) and protein contents (% dm, AOAC Official Dumas Method[19]) of the flour were 13.4% and 11.0%, respectively. All reagents were from Sigma–Aldrich (Bornem, Belgium) and of analytical grade unless specified otherwise. A thermostable α-amylase (Termamyl 120L) and a bacterial protease

(Neutrase 0.8L) were from Novozymes (Bagsvaerd, Denmark). Silica gel (Stabifix super) was from Stabifix Brauerei-Technick KG (Graefelfing/Munich, Germany).

The *B. subtilis* endoxylanase used was from Danisco (Brabrand, Denmark) and the *A. aculeatus* endoxylanase from Novozymes (Bagsvaerd, Denmark). Endoxylanases from *A. niger*, *T. viride*, *T. longibrachiatum* (pI 5.5 enzyme) and *T. longibrachiatum* (pI 9.0 enzyme) endoxylanases were obtained from Megazyme (Bray, Ireland). Table 2 summarises the characteristics of these endoxylanases. While the *A. aculeatus* endoxylanase belongs to GHF 10, all other endoxylanases in the present study belong to GHF 11.[20] On SDS-PAGE, performed on 20% (w/v) polyacrylamide gels with a PhastSystem unit (Amersham Biosciences)[21] and silver stained according to the instructions of the manufacturer, all endoxylanases appeared as single band proteins (Fig. 5). All endoxylanases were appropriately diluted in NaOAc buffer (0.1 M, pH 4.6) containing bovine serum albumin (0.5 mg/mL). Endoxylanase concentrations (mg/mL) were estimated by spectrophotometric measurement at 280 nm and by using molar extinction coefficients calculated from the protein sequences.

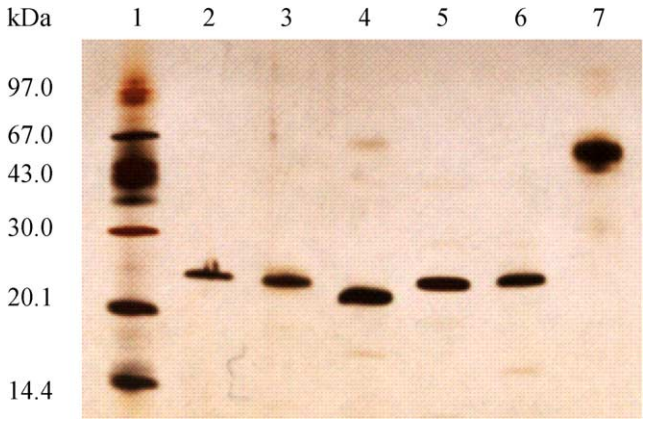


**Figure 5.** SDS-PAGE of the endoxylanases originating from *B. subtilis* (lane 2), *A. niger* (lane 3), *T. longibrachiatum* (lane 4; pI 5.5), *T. longibrachiatum* (lane 5; pI 9.0), *T. viride* (lane 6) and *A. aculeatus* (lane 7). The low-MM markers were α-lactalbumin (14.4 kDa), trypsin inhibitor (20.1 kDa), carbonic anhydrase (30.0 kDa), ovalbumin (43.0 kDa), bovine serum albumin (67.0 kDa) and phosphorylase *b* (94.0 kDa). The sizes of the MM markers (lane 1) are indicated on the left.

### 3.2. Isolation of native WE-AX from wheat flour

Native WE-AX was isolated from wheat flour as described by Cleemput et al.[22] with small adaptations. Wheat flour (200 g) was extracted with deionised water (w/v 1/5) at 4 °C (60 min). After centrifugation (3000*g*, 15 min, 4 °C), the supernatant was heated to 90 °C to precipitate soluble proteins. Residual starch was hydrolysed by addition of a thermostable α-amylase (46 μL/g flour) and the mixture was incubated at 90 °C for 30 min to degrade starch. After cooling, the mixture was centrifuged (3000*g*, 15 min, 15 °C). Silica gel was added to the supernatant as a watery suspension (20% w/v) to obtain a silica gel/protein ratio of 7. The pH of the mixture was adjusted to 3.5 for maximum adsorption of protein onto the silica gel and the mixture was incubated for 30 min under continuous stirring. After centrifugation (10,000*g*, 30 min, 15 °C), the supernatant was dialysed (48 h, 4 °C). The WE-AX in the resulting supernatant was precipitated from the solution by dropwise addition of ethanol (95%) to a final concentration of 65%. The mixture was stirred for 30 min, kept overnight at 4 °C and centrifuged (10,000*g*, 30 min, 4 °C). The WE-AX containing residue, was solubilised in water, lyophilised and ground. A WE-AX fraction (0.9 g, 86% dm WE-AX) with an A/X ratio of 0.51 was recovered with only small contaminations of glucose (5.4% dm), mannose (0.3% dm), galactose (0.5% dm) and protein (0.4% dm). Wheat endosperm WE-AX contains no glucuronic acid and is not acetylated.[23]

### 3.3. Isolation of native WU-AX from wheat flour

To isolate native WU-AX, that is, insoluble material, wheat flour was first fractionated according to MacRitchie[24] into gluten, prime starch, a squeegee fraction (SQF) and a water-extractable fraction. Wheat flour (250 g) was kneaded manually to doughs with deionised water (150 mL). Gluten was recovered by five subsequent washings with deionised water (500 mL) at room temperature. Starch [prime starch and SQF] and the water-extractable fraction were separated by centrifugation of the washing liquor (5000*g*, 10 min, 15 °C). The darker SQF layer was scraped from the white prime starch in the centrifuge tubes and was suspended in

**Table 2.** Characteristics of the endoxylanases used in this manuscript

| Enzyme source | Accession number | Reference | pI | Optimal pH | Optimal temp (°C) | $SSF_{CHROM}$[12] |
|---|---|---|---|---|---|---|
| *B. subtilis* | P18429 | Paice et al.[28] | 9.3 | 6.0–7.0 | 50 | 22.0 |
| *A. niger* | P55329 | Krengel and Dijkstra[29] | 3.5 | 3.0 | 40 | 7.9 |
| *T. longibrachiatum* | P36218 | Törronen et al.[30] | 5.5 | 4.5 | 50 | 6.4 |
| *T. longibrachiatum* | P36217 | Törronen et al.[30] | 9.0 | 6.0 | 50 | 3.4 |
| *A. aculeatus* | AAE69552 | Kofod et al.[31] | 4.5 | 4.0 | 70 | 0.9 |
| *T. viride* | AJ012718 | Megazyme product sheet E-XYTR1 | 8.4 | 4.5–5.0 | 50 | 2.7 |

deionised water. After centrifugation, the SQF was separated from the prime starch as described above. The obtained SQF contained damaged starch (81% dm), protein (1% dm) and WU-AX (6% dm).

To isolate the WU-AX from SQF, the latter was suspended in deionised water (w/v 1/10) and incubated with a thermostable α-amylase (46 μL/g SQF) at 90 °C for 30 min under continuous stirring. After centrifugation (7000*g*, 15 min, 15 °C), the residue was suspended in NaOAc (0.025 M, pH 5.0; w/v 1/3) and incubated with a protease (14 μL/g residue) at 50 °C for 20 h to remove proteins. Subsequently, the suspension was heated to 85 °C over 15 min. After cooling to room temperature, it was centrifuged (9000*g*, 15 min, 15 °C). The residue was washed with deionised water and centrifuged again. This washing step was repeated and finally the residue was suspended in ethanol (95%), dried and ground. A WU-AX fraction (1.8 g, 71% dm WU-AX) with an A/X ratio of 0.47 was obtained. It contained small levels of glucose (5.5% dm), mannose (3.5% dm), galactose (0.6% dm) and protein (3% dm). Residual cell wall material in the fraction can be assumed to be lignin or cellulose. Wheat endosperm WU-AX contain little, if any, glucuronic acid and are not acetylated.[23]

### 3.4. Incubation of WE-AX and WU-AX with endoxylanases

Wheat flour WE-AX and WU-AX were incubated with endoxylanases according to Courtin and Delcour[8] with small modifications. Thus, WE-AX (10.0 mg) was solubilised overnight in 4.5 mL NaOAc buffer (0.1 M, pH 4.6). Suitably diluted endoxylanase solution (0.5 mL) was added and the mixture was incubated at 30 °C for 15 min with continuous stirring. After centrifugation (4500*g*, 15 min, 20 °C), the supernatant was filtered and heat treated (120 °C, 30 min). WU-AX (12.0 mg) was suspended in 4.5 mL sodium acetate buffer (0.1 M, pH 4.6) and suitably diluted enzyme solutions (0.5 mL) were added. The mixture was incubated (30 °C, 15 min) under continuous stirring. After centrifugation (4500*g*, 15 min, 20 °C), the supernatant was filtered and heat treated (120 °C, 30 min). All incubation experiments were performed in triplicate for a range of 6 enzyme dilutions and a control. Supernatants were stored for analysis.

All incubation experiments were performed in the same buffer (NaOAc buffer, pH 4.6). This buffer was chosen to allow comparison of the results obtained in this manuscript with $SSF_{CHROM}$ values described earlier.[12]

### 3.5. Determination of WU-AX solubilisation

The monosaccharide composition of supernatants recovered after incubation of WU-AX with different dosages of endoxylanases was estimated following acid hydrolysis, reduction, acetylation and gas liquid chromatography (GLC) of the resulting alditol acetates.[25,26] The derivates (1.0 μL) were separated on a Supelco SP-2380 column (30 m × 0.32 mm ID, 0.2 μm film thickness, Bellefonte, PA, USA) with helium as the carrier gas, in an Agilent 6890 Series chromatograph (Wilmington, DE, USA) equipped with a flame ionisation detector. The column temperature was 225 °C, the injection and detection temperatures were 270 °C.

The solubilising activity of an endoxylanase ($A_{SX,WU\text{-}AX}$) is defined as the amount of solubilised xylose (SX) (mostly present under polymeric form, expressed as nanomole equivalent xylose) released in the supernatant per second in the presence of an excess WU-AX (as evidenced by a linear response) and under the experimental conditions used. This activity is expressed as nanokatal (nKat). Specific solubilising activity is solubilising activity per mg endoxylanase ($A_{SX,WU\text{-}AX}$/mg; nKat/mg).

### 3.6. Determination of carbohydrate reducing ends

Carbohydrate reducing ends in the supernatants recovered after incubation of WE-AX and WU-AX with different dosages of endoxylanase were determined as alditol acetates by GLC following reduction, acid hydrolysis and acetylation of the supernatants.[27]

Enzyme activity towards WE-AX or WU-AX, based on the formation of reducing xylose (RX) ($A_{RX,WE\text{-}AX}$ or $A_{RX,WU\text{-}AX}$), is the amount of RX (in nanomoles) formed per second in excess WE-AX or WU-AX (as evidenced by a linear response) and under the experimental conditions used. These activities are expressed as nanokatal (nKat). Specific enzyme activity towards WE-AX or WU-AX is enzyme activity towards WE-AX or WU-AX per mg endoxylanase ($A_{RX,WE\text{-}AX}$/mg or $A_{RX,WU\text{-}AX}$/mg; nKat/mg).

### 3.7. Determination of apparent MM of AX fragments

The apparent MM distribution of the AX fragments in the supernatants, recovered after incubation of WE-AX and WU-AX with different dosages of endoxylanase, were estimated using high-performance size exclusion chromatography (HPSEC) on a high-performance liquid chromatography system (Kontron Instruments 325 pump system, Kontron, Milan, Italy) with auto-injection. The supernatants were separated on a Shodex SB-806 HQ column (300 mm × 8 mm ID) with a Shodex SB-G guard column (50 mm × 6 mm ID) from Showa Denko K.K. (Tokyo, Japan). Elution of the samples (20 μL) was with 0.3% NaCl (0.5 mL/min at 30 °C). The refractive index of the eluent was monitored using a refractive index detector model 8110 (VSD Optilab, Berlin, Germany). MM markers were Shodex standard P-82 pullulans with MM of $78.8 \times 10^4$, $40.4 \times 10^4$, $21.2 \times 10^4$,

$11.2 \times 10^4$, $4.73 \times 10^4$, $2.28 \times 10^4$, $1.18 \times 10^4$ and $0.59 \times 10^4$ Da and glucose.

## Acknowledgements

The Instituut voor de aanmoediging van Innovatie door Wetenschap en Technologie in Vlaanderen (IWT, Brussels, Belgium) is thanked for GBOU project funding. This study was also carried out in the framework of research project GOA/03/10 financed by the Research Fund K. U. Leuven and with financial support from the Commission of the European Communities, specific RTD programme 'Quality of Life and Management of Living Resources', QLK1-2000-00324, 'Solfibread: Barley β-glucan and wheat arabinoxylan soluble fibre technologies for health promoting bread products'. It does not necessarily reflect its views and in no way anticipates the Commission's future policy in this area.

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