

## Endoxylanase Inhibition Activity in Different European Wheat Cultivars and Milling Fractions

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### ABSTRACT

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Twenty-three wheat samples from 19 different European wheat cultivars (*Triticum aestivum* L.) were tested for their quantitative and qualitative variation in inhibition activity against family 11 endoxylanases of *Aspergillus niger*, *Bacillus subtilis*, and *Trichoderma viride* and a family 10 endoxylanase of *A. aculeatus*. Under the experimental conditions, the *A. aculeatus* enzyme was not inhibited by the wheat extracts, the *A. niger* and *B. subtilis* endoxylanases were affected to a similar extent, while the *T. viride* enzyme was much more inhibited. The inhibition activities in the different wheat samples against the *A. niger*, *B. subtilis*, and *T. viride* endoxylanases varied between 36.0 and 11.7, 34.0 and 12.9, and 86.2 and 46.6 IU/100 mg of dry whole meal, respectively. One IU (inhibition unit) corresponds to the amount of inhibitor resulting in 50% inhibition of endoxylanase activity under the conditions of the assay. The inhibitor

activities were linearly related, indicating that the levels of different endoxylanase inhibitors with different endoxylanase specificities in the dormant wheat grains are also linearly related or that one (or more) of these inhibitors are predominantly present or has much higher specific activity, consequently causing almost all of the inhibition activity measured. Wheat flour accounted for ≈57% of the total inhibition activity in wheat grains, while the shorts and bran fractions each contained ≈21% of the total activity. On dry weight basis, the inhibition activities were about three times higher in shorts and about two times higher in bran than in flour. The results obtained may be useful in explaining differences in functionality of different endoxylanases in biotechnological processes in which wheats of different cultivars, or fractions thereof, are used as well as in screening endoxylanases for applications in wheat-based processes.

The most abundant wheat (*Triticum aestivum* L.) kernel non-starch polysaccharides are arabinoxylans (AX). Their structure and physicochemical characteristics affect their functionality and that of wheat in different biotechnological processes and applications. Obvious examples are the breadmaking process (Courtin et al 1999), refrigerated dough systems (Poulsen and Sørensen 2001), the gluten starch separation (Christophersen et al 1997), the production of white beers (Debyser 1999), and animal feeding (Bedford and Classen 1992). AX can be degraded by several enzymes including endo-β-1,4-xylanases (endoxylanases, EC 3.2.1.8), β-D-xylosidases (EC 3.2.1.37) and α-L-arabinofuranosidases (EC 3.2.1.55) (Biely 1985). From a biotechnological point of view, endoxylanases are the most important xylanolytic enzymes because they have a profound impact on the functionality of AX and wheat in the cited processes (Courtin et al 1999, 2001; Weegels et al 1992; Christophersen et al 1997) and, hence, have a widespread use today.

A recent insight is that wheat and other cereals such as rye (*Secale cereale* L.) and barley (*Hordeum vulgare* L.) (Debyser and Delcour 1997; McLauchlan et al 2000; Gebruers et al 2001; Goesaert et al 2001; Goesaert et al, *in press*) contain proteinaceous inhibitors of endoxylanases. To date, two main types of endoxylanase inhibitors with different structures and endoxylanase specificities have been described in literature. The first type are the high pI and non-glycosylated TAXI [*Triticum aestivum* (endo)xylanase inhibitor]-like inhibitors with a molecular mass of ≈40,000 and pI values of at least 8.8 (Debyser and Delcour 1997; Gebruers et al 2001; Goesaert et al 2001; Goesaert et al, *in press*). TAXI proteins occur in two different molecular forms, one of which is proteolytically derived from the other one (Debyser and Delcour 1997; Debyser et al 1999). Quite recently, our group found at least two TAXI proteins in wheat (TAXI I and TAXI II), with different capacities to inhibit endoxylanases (Gebruers et al 2001). The second type are the XIP (xylanase

inhibiting protein)-like inhibitors, which have also a high pI but have a different structure. They are monomeric, glycosylated proteins and of lower molecular weight (≈30,000) (McLauchlan et al 1999; Hessing and Happe 2000).

At present, the functionality of endoxylanase inhibitors in wheat and in the biotechnological processes and applications in which the cereal is used, is poorly understood. Enzymic modification of the AX population by endoxylanases can indeed have a positive impact on these processes and applications or final product quality. However, in many cases, discrepancy exists between the *in vitro* degradation of AX by endoxylanases and the effect of these enzymes in the real life processes and applications. Furthermore, different responses to endoxylanases by different wheat flours have also been noticed (Rouau et al 1994; Classen et al 1995). This might partially be explained by the presence of endoxylanase inhibitors and the quantitative and qualitative variation in inhibition activity in wheat grains of different cultivars.

To contribute more insight in this novel area, we studied the quantitative and qualitative variation in inhibition activity in different European wheat cultivars and milling fractions.

### MATERIALS AND METHODS

Twenty-three different wheat samples from 19 different European cultivars were supplied by AVEVE (Landen, Belgium) and Cargill (Bergen-op-Zoom, The Netherlands) (Table I). Whole meals were obtained using a Cyclotec 1093 sample mill (Tecator, Hogånäs, Sweden) and the eight different wheat milling fractions were obtained with a Bühler MLU-202 mill (Uzwill, Switzerland).

The endoxylanases from *Aspergillus niger* (M4) and *Trichoderma viride* (M1), were from Megazyme (Bray, Ireland) while those from *Bacillus subtilis* and *Aspergillus aculeatus* were supplied by Puratos (Groot-Bijgaarden, Belgium). The chromogenic azurine cross-linked wheat AX substrate tablets were from Megazyme. Bovine serum albumin (BSA) was from Sigma-Aldrich (Bornem, Belgium). All other reagents used were from Sigma-Aldrich and of analytical grade, unless specified otherwise.

The moisture contents of the whole meals and the wheat milling fractions were determined according to Approved Method 44-19 (AACC 2000). All measurements were performed at least in duplicate.

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### Total and Soluble Protein Content

All protein contents ( $N \times 5.7$ ) were determined with the Dumas method according to AOAC method 992.23.

For albumin protein contents, the wheat whole meals or milling fractions (2.0 g) were extracted at room temperature for 2 hr with water (20 mL) while the same quantity was extracted under similar conditions with 0.5M NaCl (20 mL) for estimation of albumin plus globulin protein contents (Chen and Bushuk 1970). The extracts were centrifuged ( $5,000 \times g$ , 30 min, 7°C). Small quantities of whole meals (300 mg), milling fractions (300 mg), or supernatants (1.0 mL) were analyzed with the vario MAX CN (Elementar Laboratory Technology, Gouda, The Netherlands) using O<sub>2</sub> flows of 75, 75, and 100 mL/min respectively. All measurements were performed at least in triplicate.

### Endoxylanase Inhibition Assay

Inhibition activities were determined with the colorimetric xylazyme-AX method based on the release of soluble dyed fragments of azurine cross-linked wheat AX (Gebruers et al 2001). Wheat whole meal or wheat milling streams (2.0 g) were extracted for 30 min

with sodium acetate buffer (25 mM, pH 5.0, 20 mL) and centrifuged ( $10,000 \times g$ , 30 min, 7°C). The supernatants were heated (40 min, 65°C) and diluted 12.5× (*A. aculeatus*, *A. niger*, and *B. subtilis* endoxylanases) or 25× (*T. viride* endoxylanase) before measuring inhibition activity. The very strong inhibition activity against *T. viride* endoxylanase required greater dilution. All endoxylanase solutions were prepared in sodium acetate buffer (25.0 mM, pH 5.0) with BSA (0.5 mg/mL) and contained 2.0 enzyme units/1.0 mL, 1.0 enzyme unit corresponding to an increase in A<sub>590</sub> (absorbance at 590 nm) of 1.0 in the xylazyme-AX method in absence of inhibitor. For all enzymes used, we ascertained that, under the conditions of the assay, there was a linear response between the quantity of cereal extract and the resulting enzyme inhibition.

Endoxylanase solution (0.5 mL) was preincubated for 30 min at room temperature with an equal amount of diluted wheat sample extract or buffer (control). The mixture was subsequently kept at 30°C and, after 10 min, an azurine cross-linked wheat AX tablet was added. It was then incubated for 60 min at 30°C. The reaction was terminated by adding 1.0% (w/v) Tris[hydroxymethyl]-amino-methane solution (10.0 mL) and vigorous vortex stirring. After 10 min

**TABLE I**  
Relative Inhibition Activities (IU/100 mg, dry weight basis)<sup>a</sup> Against *Aspergillus niger* (RIA<sub>DW</sub><sup>A.niger</sup>), *Trichoderma viride* (RIA<sub>DW</sub><sup>T.viride</sup>), and *Bacillus subtilis* (RIA<sub>DW</sub><sup>B.subtilis</sup>) Endoxylanases in Different European Wheat Cultivars

Wheat Cultivar	RIA <sub>DW</sub> <sup>A. niger</sup> (IU/100 mg)	RIA <sub>DW</sub> <sup>B. subtilis</sup> (IU/100 mg)	RIA <sub>DW</sub> <sup>T. viride</sup> (IU/100 mg)	Total Protein (%)	Albumin (%)	Albumin + Globulin (%)
Cadenza A	36.0	34.0	86.2	14.8	3.4	5.1
Cadenza B	28.9	29.3	73.1	12.4	3.3	4.6
Ritmo A	32.2	28.8	77.6	12.5	3.0	4.6
Ritmo B	31.7	31.2	80.8	11.4	2.9	4.5
Versailles A	30.4	29.7	75.3	12.9	nd <sup>b</sup>	4.7
Versailles B	32.0	32.3	77.4	10.4	nd	4.0
Vivant A	29.9	26.3	72.2	11.9	nd	4.8
Vivant B	30.1	26.5	70.9	11.5	nd	4.7
Semper A	28.9	27.1	71.9	12.3	nd	4.4
Harrier A	27.8	24.7	73.3	12.9	nd	5.2
Farandole A	29.3	28.3	78.8	14.0	nd	4.7
Anemos A	26.9	25.2	65.3	14.0	3.3	4.0
Residence A	29.2	24.6	71.8	11.9	3.1	4.5
Bercy A	27.9	25.4	71.1	13.3	3.5	5.0
Cezanne A	11.7	12.9	46.6	13.6	3.1	4.5
Tremie A	28.3	28.9	71.3	11.4	nd	4.5
Rialto A	31.6	26.9	72.7	12.0	3.8	4.7
Reaper A	29.1	29.2	71.8	10.8	nd	4.3
Poseidon A	24.5	25.1	68.1	13.7	3.2	4.6
Soissons A	25.8	22.6	59.1	13.8	3.0	4.6
Cyrano A	21.9	18.2	58.0	11.3	2.8	4.1
Baldus A	18.6	14.6	51.6	11.5	2.1	4.2
Record A	23.8	21.0	59.5	11.8	3.0	4.2
EE <sup>c</sup>	<3%	<3%	<4%	<3%	<4%	<4%

<sup>a</sup> 1.0 IU = amount of inhibitor that, under the experimental conditions, results in 50% inhibition of an endoxylanase.

<sup>b</sup> Not determined.

<sup>c</sup> Experimental error.

**TABLE II**  
Yields of Different Milling Fractions (% dry weight) of Ritmo B Wheat Sample with Levels (% dry weight) of Total Protein and Relative Inhibition Activities (IU/100 mg)<sup>a</sup> Against *Aspergillus niger* and *Bacillus subtilis* Endoxylanases on Dry Weight (RIA<sub>DW</sub>) and Total Protein (RIA<sub>TP</sub>) Basis

Sample <sup>b</sup>	Milling Fraction (%)	Total Protein (%)	<i>A. niger</i> Endoxylanase (IU/100 mg)		<i>B. subtilis</i> Endoxylanase (IU/100 mg)	
			RIA <sub>DW</sub>	RIA <sub>TP</sub>	RIA <sub>DW</sub>	RIA <sub>TP</sub>
B1	17.8	8.6	22.7	265	19.6	229
B2	8.4	11.8	26.7	227	24.1	204
B3	1.4	14.0	28.9	206	26.7	191
C1	38.5	9.6	24.5	255	20.5	213
C2	7.8	10.5	27.1	257	25.0	237
C3	1.4	12.1	28.0	231	26.7	221
Flour	75.3	9.8	24.7	252	21.4	218
S	9.8	15.6	70.8	454	65.1	417
B	15.0	15.3	48.1	314	41.0	268
Meal	100.0	11.2	32.7	334	28.6	255

<sup>a</sup> 1.0 IU = amount of inhibitor that, under experimental conditions, results in 50% inhibition of an endoxylanase.

<sup>b</sup> B1, B2, and B3 are break fractions 1, 2 and 3, respectively; C1, C2, and C3 are reduction fractions 1, 2, and 3, respectively; S is shorts; and B is bran.

at room temperature, the tube was shaken vigorously and the content was filtered through a Schleicher & Schuell filter ( $\phi$  90 mm) (Dassel, Germany). The  $A_{590}$  value was measured with an Ultraspec III UV/Visible spectrophotometer (Pharmacia Biotech, Uppsala, Sweden) against a control prepared by incubating the sample with buffer instead of enzyme solution.

The relative inhibition activities (RIA) were expressed as a number of inhibition units (IU), the amount of inhibitor resulting in 50% inhibition of endoxylanase activity under the conditions of the assay/100 mg of dry weight or total protein, referred to as  $RIA_{DW}$  and  $RIA_{TP}$ , respectively. All analyses were at least in triplicate.

## RESULTS AND DISCUSSION

### Inhibition Activities in Different Wheat Cultivars

Under the experimental conditions used, the glycosyl hydrolase family 10 *A. aculeatus* endoxylanase was not inhibited by any of the wheat extracts. However, the glycosyl hydrolase family 11 endoxylanases of *A. niger*, *B. subtilis*, and *T. viride* were all inhibited. Under experimental conditions, the  $RIA_{DW}$  values against *A. niger* ( $RIA_{DW}^{A.niger}$ ), *B. subtilis* ( $RIA_{DW}^{B.subtilis}$ ), and *T. viride* ( $RIA_{DW}^{T.viride}$ ) endoxylanases of the tested European wheat cultivars varied widely, at 36.0–11.7, 34.0–12.9, and 86.2–46.6 IU/100 mg, respectively. For these three endoxylanases, the lowest activities were measured in the Cezanne wheat sample, while the highest activities were obtained for the Cadenza wheat sample (sample A). For the Cadenza wheat samples, we noticed significantly higher  $RIA_{DW}^{A.niger}$ ,  $RIA_{DW}^{B.subtilis}$  and  $RIA_{DW}^{T.viride}$  values for sample A than for sample B, while for the Ritmo, Versailles, and Vivant samples, no significant differences could be observed. The differences with the Cadenza samples might be explained by differences in climatological and soil conditions, time of harvest, or storage conditions.

The  $RIA_{DW}^{A.niger}$ ,  $RIA_{DW}^{B.subtilis}$  and  $RIA_{DW}^{T.viride}$  values of the different wheat samples (Table I) were linearly related (Fig. 1). The endoxylanases of *A. niger* and *B. subtilis* were similarly affected by the wheat wholemeal extracts (Fig. 1A, Table I), while the enzyme of *T. viride* was 2.3–4.0 $\times$  more inhibited (Fig. 1B and C, Table I). These findings may indicate that the levels of different endoxylanase inhibitors with different endoxylanase specificities in the dormant wheat grains are also linearly related or that one (or more) of these inhibitors is predominantly present or has much higher specific activity, consequently causing almost all of the inhibition activity measured. The specificities of the endoxylanase inhibitors can be summarized as: 1) TAXI I inhibitor inhibits all three family 11 endoxylanases, but has the highest activity against the *A. niger* and *T. viride* endoxylanases; 2) TAXI II inhibitor only inhibits the *B. subtilis* and *T. viride* endoxylanases, the latter however to a much higher degree than the former (Debyser et al 1999; Gebruers et al 2001); and 3) XIP inhibitor only inhibits the fungal family 11 endoxylanases, that is those of *A. niger* and *T. viride*, and this to a similar degree (McLauchlan et al 1999). This might explain the stronger inhibition of the *T. viride* endoxylanase by the wheat extracts than that of other endoxylanases, as it is inhibited by all three inhibitor types. As the *A. niger* and *B. subtilis* endoxylanases were similarly inhibited, TAXI II-like endoxylanase inhibitor probably compensates the low  $RIA_{DW}^{B.subtilis}/RIA_{DW}^{A.niger}$  ratio of TAXI I and XIP.

No significant correlation ( $P > 0.05$ ) could be detected between the  $RIA_{DW}^{A.niger}$ ,  $RIA_{DW}^{B.subtilis}$  and  $RIA_{DW}^{T.viride}$  values and the globulin (estimated by the difference between albumin plus globulin and albumin protein contents), albumin plus globulin, and total protein contents of the different wheat samples. Whereas the  $RIA_{DW}^{A.niger}$  and  $RIA_{DW}^{T.viride}$  values were not significantly related to albumin protein contents ( $P > 0.05$ ), the  $RIA_{DW}^{B.subtilis}$  values were ( $P = 0.04$ ), although weak.

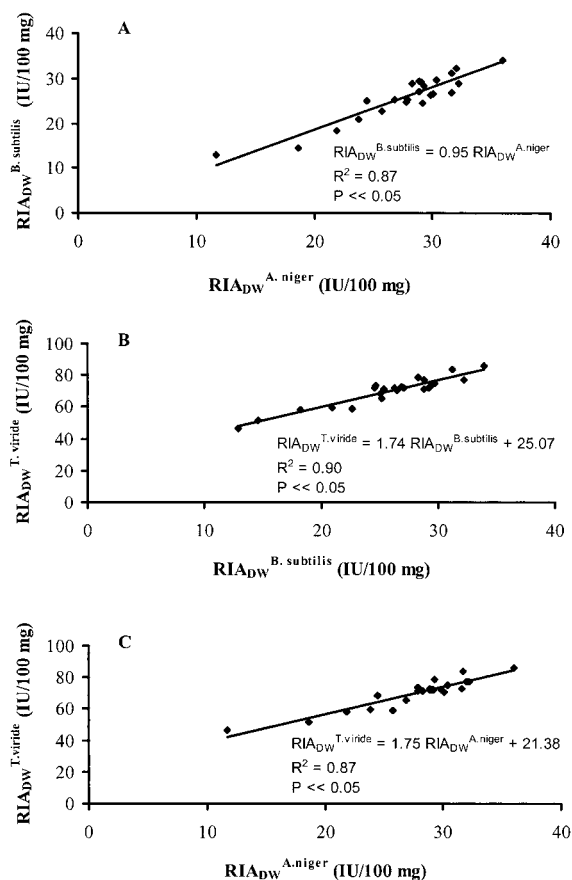
### Inhibition Activities in Different Milling Fractions

Milling of Ritmo B wheat grains resulted in 75% flour (break fractions B1 to B3 and reduction fractions C1 to C3) (Table II),

which contained  $\approx 57\%$  of the total inhibition activity against the endoxylanases of *A. niger* and *B. subtilis* present in the grains. The shorts (S fraction, Table II) and the bran (B fraction, Table II) made up  $\approx 10$  and 15% of the wheat kernels and each contained  $\approx 21\%$  of the total inhibition activity of the grains. The  $RIA_{DW}$  values decreased in the order of shorts, bran, and flour. Values were about three times higher in shorts and about two times higher in bran than in flour. Furthermore, for the different flour fractions, the  $RIA_{DW}$  values increased from the first to the third break fraction and from the first to the third reduction fraction, those of the break fractions were comparable with those of the reduction fractions. In spite of the higher protein concentration in shorts and bran, the  $RIA_{TP}$  values of shorts were still higher than those of bran, and the latter still higher than those of the flour fractions (Table II).

### Relevance of Present Findings

The dosages of enzymes used in the present work are of the same order of magnitude or even higher than those which, in many instances, are used to the advantage of process or final product quality parameters in biotechnological processes and applications in which cereals and their milling streams are used. Consequently, it could be argued that the effect of the wheat inhibitors on endoxylanase activity during these processes are at least as pronounced as described above. Therefore, the present data not only indirectly show that, depending on the enzyme, endoxylanase functionality may be influenced by the presence of endoxylanase inhibitors, but also that it is affected to different degrees by different wheat cultivars and



**Fig. 1.** Correlation between relative inhibition activities (IU/100 mg, on dry weight basis) against *Aspergillus niger* ( $RIA_{DW}^{A.niger}$ ), *Trichoderma viride* ( $RIA_{DW}^{T.viride}$ ), and *Bacillus subtilis* ( $RIA_{DW}^{B.subtilis}$ ) endoxylanases in different European wheat cultivars. One IU corresponds to the amount of inhibitor resulting in 50% inhibition of endoxylanase activity under the conditions of the assay.

milling fractions. It follows that part of the variance in efficiency of a given endoxylanase in a particular process may be directly related to the level of inhibitors present in the wheat raw material used, but also that differences in efficacy of different endoxylanases may be directly related to their susceptibility to inhibition.

According to Debyser et al (1997), AX solubilization by barley malt associated endoxylanases during the production of Belgian white beers is significantly reduced by the use of an unmalted wheat adjunct. Sibbesen and Sørensen (2000, 2001) observed that endoxylanase inhibitors can have a significant effect on the impact of endoxylanases on dough stickiness, AX solubility and molecular weight, dough handling properties, and dough slurry viscosity. Furthermore, Poulsen and Sørensen (2001) demonstrated that the addition of extra endoxylanase inhibitor to flour decreased the amount of brownish liquid (syrup) leaking from dough during storage. This phenomenon, called syrupeing, is caused by the extensive breakdown of AX by wheat flour associated endoxylanases. The present findings hold promise for the development of endoxylanases that are insensitive to endoxylanase inhibitors (Gravesen and Derkx 2001) and result in a lower endoxylanase dosage in the processing of cereals.

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