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TAXI Type Endoxylanase Inhibitors in Different Cereals

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An affinity-based purification procedure with the immobilized family 11 *Bacillus subtilis* endoxylanase XynA allowed us to obtain high yields of highly pure endoxylanase inhibitor fractions from rye, barley, and durum wheat. In contrast, no inhibitors interacting with the *B. subtilis* endoxylanase affinity column are present in corn, buckwheat, rice, and oats. Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis and inhibitor specificity showed that the isolated inhibitors belonged to the TAXI endoxylanase inhibitor family, thus providing a view on the diversity of this cereal inhibitor family. The isolated inhibitors are basic proteins of ca. 40 kDa, occurring in two molecular forms, with pI values of ca. 8.5 (durum wheat) and ca. 9.0 (rye, barley). They are, in general, strong inhibitors of family 11 endoxylanases but not of family 10 endoxylanases. Because cereal endogenous endoxylanases belong to the latter family, this probably indicates that they do not influence cereal metabolic processes. For the first time, endoxylanase inhibitors, similar to TAXI I and TAXI II from wheat, were isolated from durum wheat and characterized. For each cereal, high-resolution cation exchange chromatography revealed the presence of multiple isoinhibitors, each of which occurs in two molecular forms. However, in durum wheat and barley, a single isoform is predominantly present.

KEYWORDS: Endoxylanase inhibitor; cereals; TAXI; affinity chromatography

INTRODUCTION

Arabinoxylans, dietary fiber constituents present in cereal cell walls, are one of the main quality determining factors in several cereal-based biotechnological processes. They have an impact on bread making (*1*, *2*) and wheat gluten/starch separation (*3*), and they affect animal feed performance (*4*, *5*). Endoxylanases, the most important enzymes in the hydrolysis of these nonstarch polysaccharides, are used in many food and feed applications to modify or degrade the arabinoxylan population. Such hydrolysis improves the processability and/or end use quality of some cereal products (*2*, *6*, *7*). However, recent studies have shown that cereals, such as wheat, durum wheat, rye, and barley, contain proteins capable of effectively inhibiting endoxylanases (*8*−*10*), thus affecting the functionality and performance of many of these enzymes (*11*).

In the recent literature, two types of endoxylanase inhibitors with different structures and specificities have been described, i.e., TAXI type and the even more recent XIP type inhibitors. TAXI type inhibitors (*9*, *12*−*16*) are basic proteins of ca. 40 kDa and occur in two molecular forms, i.e., a monomeric form, consisting of a single polypeptide chain with at least one intramolecular disulfide bridge, and a heterodimeric form, consisting of two disulfide-linked subunits of ca. 30 and ca. 10 kDa. The latter form presumably originates from the former following proteolytic modification (*9*, *12*, *14*−*16*). In general, the TAXI type inhibitors are active against endoxylanases of glycosyl hydrolase family 11 and show no activity toward family 10 endoxylanases (*14*−*16*). On the basis of their varying activities toward family 11 endoxylanase Xyn1 from *Aspergillus niger*, two different types of TAXI proteins, i.e., TAXI I and TAXI II, can be distinguished in wheat (*14*). The properties of HVXI and the different SCXIs, the barley and rye TAXI type inhibitors, respectively, are similar to those of TAXI I from wheat (*15*, *16*).

XIP type inhibitors from wheat and rye are basic, monomeric, glycosylated proteins of ca. 30 kDa (*17*−*19*). XIP specifically and competitively inhibits fungal endoxylanases (apart from the *Aspergillus aculeatus* family 10 endoxylanase) and is inactive against bacterial enzymes (*20*).

In the above studies, elaborate purification procedures, including CEC and GPC, were used to isolate the TAXI type inhibitors. However, we recently developed an alternative purification method based on AC with immobilized endoxylanases for the purification of several wheat isoinhibitors (*21*, *22*). This aided in obtaining a clear image of the different endoxylanase inhibitors in wheat. In contrast, knowledge about the occurrence and properties of nonwheat endoxylanase inhibitors is rather limited.

Preliminary experiments revealed a large qualitative and quantitative variation in inhibition activity between several cereals. The highest levels of inhibition activity occurred in wheat and rye whole meal extracts. Depending on the enzyme, the durum wheat inhibitor levels are ca. 15−30% of those of wheat, while barley and corn contained 5−10 and 0−2%, respectively, of the inhibition activity found in wheat (*23*). To elucidate the

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observed variation in inhibition activity in cereals, in the present study, AC was used for the fractionation and characterization of cereal endoxylanase inhibitors, in particular those belonging to the TAXI inhibitor family. The properties of the isolated inhibitors are compared with those of the already known TAXI type inhibitors. This way, we provide an overview of the biodiversity and properties of the nonwheat members of this inhibitor family.

MATERIALS AND METHODS

Chemicals. All reagents were of at least analytical grade and supplied by Sigma-Aldrich (Bornem, Belgium) unless specified otherwise. Azurine cross-linked wheat arabinoxylan tablets (Xylazyme AX) were purchased from Megazyme (Bray, Ireland). All chromatographic and electrophoresis media and markers were from Amersham Biosciences (Uppsala, Sweden).

Enzymes. The family 11 endoxylanases from XTV (molecular mass (MM) 20.0 kDa; pI 8.4; optimal pH 4.5−5.0), XTL2 and XTL3 (SWISS-PROT accession number P36218; MM 19.0 kDa; pI 5.5; optimal pH 4.5; and SWISS-PROT accession number P36217; MM 21.0 kDa; pI 9.0; optimal pH 6.0, respectively), and XAN (SWISS-PROT accession number P55329; MM 20 kDa; pI 3.5; optimal pH 3.5) were purchased from Megazyme. The family 11 XBS (mutant of XynA; MM 20.3 kDa; pI 9.3; optimal pH 6.0−7.0) and the family 10 XAA (NCBI accession number AAE69552; MM 56 kDa; pI 4.5; optimal pH 4.0) were kindly made available by Puratos (Groot-Bijgaarden, Belgium). The family 10 XANid (SWISS-PROT accession number Q00177; MM 34 kDa; pI 3.4; optimal pH 6.0) was kindly offered by the Institute of Food Research (Norwich, U.K.). The affinity column was prepared by immobilizing the endoxylanase isolated from Grindamyl H 640 (i.e., XBS, SWISS-PROT accession number P18429; Danisco Cultor, Brabrand, Denmark) (cf. infra).

Cereals. Barley (*Hordeum vulgare* L. cv. Majestic), rye (*Secale cereale* L. cv. Halor), and oats (*Avena sativa* L. cv. Evita) were obtained from Aveve (Landen, Belgium). Brown rice (*Oryza sativa* L. cv. Puntal) was from Masterfoods (Olen, Belgium). Durum wheat (*Triticum durum* Desf. Cv. Avonlea) was supplied by the Canadian Grain Commission (Winnipeg, Canada). Corn (*Zea mays*) was a commercial sample from Derboven (Wijgmaal, Belgium). Buckwheat (*Fagopyrum esculentum* Moench) groats were from Koopmans (Leeuwarden, The Netherlands). Each cereal was ground into whole meal using a Cyclotec 1093 sample mill (Tecator, Hogänäs, Sweden).

Preparation of the Affinity Column.

Endoxylanase Purification. XBS was purified from the Grindamyl H 640 bakery enzyme preparation as described elsewhere (*21*). Following extraction of the preparation and centrifugation, the purification consisted of a single CEC step on a SP Sepharose Fast Flow column (26 mm × 300 mm), equilibrated with a 25 mM sodium acetate buffer (pH 4.0). The endoxylanase was eluted with a linear gradient (0−0.5 M NaCl in 800 mL, flow rate of 5.0 mL/min), dialyzed against deionized water (48 h, 6 °C), and finally freeze-dried.

Endoxylanase Immobilization. The purified endoxylanase was linked to a *N*-hydroxysuccinimide-activated Sepharose 4 Fast Flow matrix, according to the manufacturer's instructions (*21, 22*), using 10−15 mg of enzyme for 1.0 mL of matrix. The coupling reaction was performed in a 200 mM $NaHCO_3$ buffer (pH 8.3; ca. 7.0 mL) containing 0.5 M NaCl and was allowed to proceed for 2.5 h at room temperature while the mixture was shaken continuously. After excess endoxylanase was removed by rinsing with 0.5 M ethanolamine (pH 8.3) containing 0.5 M NaCl (35 mL), the matrix was incubated with the same ethanolamine solution and shaken vigorously for 4 h at room temperature. The matrix was then washed with a 0.1 M glycine solution (pH 3.0) containing 0.5 M NaCl (35 mL) and with the above ethanolamine solution (35 mL). It was then equilibrated with 25 mM sodium acetate buffer (pH 5.0) containing 0.2 M NaCl (70 mL). In this way, an affinity column (10 mm × 70 mm) with XBS as ligand was obtained.

Endoxylanase Inhibition Assay Procedure. The endoxylanase inhibition activity was determined colorimetrically with the Xylazyme AX method (Megazyme product sheet Xyl 7/01) as described previously (*15*). In this procedure, (diluted) inhibitor (0.5 mL) and enzyme (0.5 mL) solutions, prepared in a 25 mM sodium acetate buffer (pH 5.0), were incubated for 30 min at room temperature and an additional 10 min at 30 °C prior to addition of the Xylazyme AX substrate tablets. The reaction proceeded for 60 min at 30 °C and was then stopped by addition of 10.0 mL of 1% (w/v) Tris solution and vigorous vortex stirring. After 10 min at room temperature, the suspension was shaken, and the absorbance at 590 nm (A_{590}) of the filtrate was measured. Inhibition activity was expressed as the reduction (in %) of the endoxylanase activity, determined in the absence of inhibitor. One InU was defined as the inhibitor amount that under the conditions of the assay, reduced a fixed endoxylanase activity with 50%. Before use, all endoxylanases were appropriately diluted in a sodium acetate buffer (25 mM, pH 5.0) containing 0.05% (w/v) BSA to stabilize the enzymes. Under the conditions of the assay, the enzyme concentrations corresponded to an increase in A_{590} of 1.0 and were ca. 230, ca. 270, ca. 82, ca. 132, and ca. 100 ng/mL, ca. 5 μg/mL, and ca. 900 ng/mL for XAN, XBS, XTV, XTL2, XTL3, XAA, and XANid, respectively. All measurements were made in duplicate, unless specified otherwise.

Isolation of Endoxylanase Inhibitors from Different Cereals.

Preparation of Crude Cereal Whole Meal Extracts. Each cereal whole meal (500 g) was extracted with 25 mM sodium acetate buffer (pH 5.0) (1:5 w/v, except for rye and oats where a 1:8 w/v ratio was used) for 30 min at 6 °C by mechanical shaking. After they were centrifuged (5000*g*; 30 min; 6 °C), the supernatants were filtered. Finally, the pH of the filtrates was adjusted to 5.0 with 1.0 M HCl.

Concentration of the Cereal Extracts by CEC. The cereal extracts were applied at a flow rate of 4 mL/min to a SP-Sepharose Fast Flow Column (26 mm × 300 mm), equilibrated with a 25 mM sodium acetate buffer (pH 5.0). Under these conditions, proteins with endoxylanase inhibition activity are retained. They were then eluted in one step with 1.0 M NaCl (500 mL). The eluates were dialyzed against deionized water (48 h; 6 °C) and lyophilized, yielding the CEC fraction.

Inhibitor Purification by AC with Immobilized XBS. The different CEC fractions were dissolved in a 25 mM sodium acetate buffer (pH 5.0), containing 0.2 M NaCl (buffer A) (final concentration of ca. 15 mg CEC fraction/mL) and further separated by AC with the XBS affinity column, previously equilibrated with the same buffer. Following sample application (at a flow rate of 0.33 mL/min) and reequilibration with buffer A (35 mL), elution of bound inhibitor proteins was first with 7.0 mL of a 200 mM Tris solution (pH 11.0). The column was then reequilibrated with buffer A (13 mL), and the inhibitor proteins that remained on the column were next eluted with 7.0 mL of 200 mM sodium phosphate buffer (pH 12.0). Finally, the column was rinsed with buffer A (20 mL). Peak fractions (according to A_{280}) were pooled and immediately subjected to a buffer exchange (25 mM sodium acetate buffer, pH 5.0), using a PD-10 column (10 mm × 50 mm), according to the manufacturer's instructions. In this way, for each cereal, two fractions further referred to as AC−XBS11 and AC−XBS12 were obtained.

Protein Determination. Protein concentrations were estimated by the Coomassie Brilliant Blue method of Bradford (*24*) using BSA as standard.

Protein Electrophoresis. Isolated inhibitor samples (0.1−2.0 μg of protein) with or without the reducing agent 2-mercaptoethanol (5% (v/v)) were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE) on 20% polyacrylamide gels with a PhastSystem unit (Amersham Biosciences), according to Laemmli (*25*). Low MM markers (LMW marker kit, 14.4−97.0 kDa; Amersham Biosciences) were α-lactalbumin (14.4 kDa), trypsin inhibitor (20.1 kDa), carbonic anhydrase (30.0 kDa), ovalbumin (43.0 kDa), albumin (67.0 kDa), and phosphorylase b (97.0 kDa). The pI was determined by IEF with the PhastSystem using 5% polyacrylamide gels containing ampholytes (pH 3.0−9.0) and appropriate standards (Broad pI kit, pI 3.5−9.3; Amersham Biosciences). All gels were silver-stained according to instructions of the manufacturer (Amersham Biosciences Development Technique file no. 210).

Analysis of Inhibitor Fractions by HR-CEC. The endoxylanase inhibitors in the AC−XBS fractions were fractionated by CEC on a MonoS HR 5/5 column (5 mm × 50 mm), equilibrated with a 20 mM sodium phosphate buffer (pH 6.0). Bound proteins were eluted using

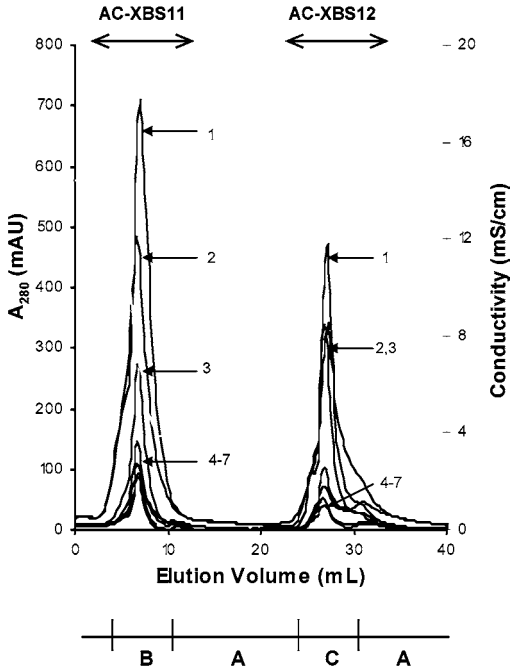

Figure 1. AC of the cereal CEC fractions on the XBS affinity column, equilibrated with a 25 mM sodium acetate buffer (pH 5.0) containing 0.2 M NaCl (**A**) showing the UV absorbance (—) and the conductivity (—). Bound inhibitors were eluted with 200 mM Tris solution (pH 11.0) (**B**) and, following reequilibration, with 200 mM sodium phosphate buffer (pH 12.0) (**C**), resulting in the AC–XBS11 and AC–XBS12 fractions, respectively. (1) Rye, (2) durum wheat, (3) barley, (4) corn, (5) rice, (6) oats, and (7) buckwheat.

a linear gradient of 0.0−0.5 M NaCl in 100 mL (flow rate 1.0 mL/min). Fractions (1.0 mL) were collected and assayed for their ability to inhibit XAN and XBS.

RESULTS AND DISCUSSION

Inhibitor Purification. In a first step, the cereal extracts were separated by CEC to reduce their volume as well as to carry out a basic, crude purification step. Next, the different CEC fractions were loaded on an affinity column to which XBS was coupled. Proteins interacting with this column were successively eluted with two alkaline buffers, i.e., first with a Tris solution of pH 11.0 and second (after reequilibration of the affinity column) with a phosphate buffer of pH 12.0. Optimization of the elution conditions was based on preliminary experiments, showing that the inhibitors and endoxylanase do not interact under alkaline conditions. However, because the Tris solution (pH 11) did not remove all inhibiting proteins bound to the XBS affinity column, a second elution step with a phosphate buffer (pH 12) was included in the purification method.

This procedure yielded two inhibitor fractions for each cereal, i.e., AC−XBS11 and AC−XBS12. In this way, high yields of rye, durum wheat, and barley endoxylanase inhibiting proteins were isolated (**Figure 1** and **Table 1**). Large inhibitor quantities (6.3 mg of protein in total) were found in both rye AC−XBS fractions, corresponding to ca. 59% of the initial inhibition activity against XBS in aqueous extracts of rye. Durum wheat and barley yielded less endoxylanase inhibiting proteins. When the yields of the durum wheat and barley AC−XBS11 and AC−XBS12 fractions are compared, most or all of the inhibitors interacting with XBS were recovered in the AC−XBS12 inhibitor fractions (durum wheat, ca. 1.2 mg of protein; barley, ca. 1.3 mg of protein). The endoxylanase inhibition activity of the durum wheat and barley AC−XBS inhibitor fractions was ca. 32 and ca. 61%, respectively, of the activity against XBS present in the initial extract (**Table 1**).

Table 1. Purification of TAXI Type Endoxylanase Inhibitors from Cereal Whole Meals[a]

cereal	fraction	total protein (mg)	total activity (InU)[b]	specific activity (InU/mg)[b]	recovery (%)
rye	crude extract	6100	71 500	12	100
	CEC	921	60 000	65	84
	AC–XBS11	3.7	42 100	6700	59
	AC–XBS12	2.6			
durum wheat	crude extract	3300	40 000	12	100
	CEC	507	21 000	41	42
	AC–XBS11	0.5	12 600	7400	32
	AC–XBS12	1.2			
barley	crude extract	2070	6200	3	100
	CEC	308	5800	19	94
	AC–XBS11	∼0	3800	3000	61
	AC–XBS12	1.3			

[a] Inhibition activities were determined against XBS. [b] One InU is the amount of inhibitor that under the assay conditions inhibits a fixed XBS activity by 50%.

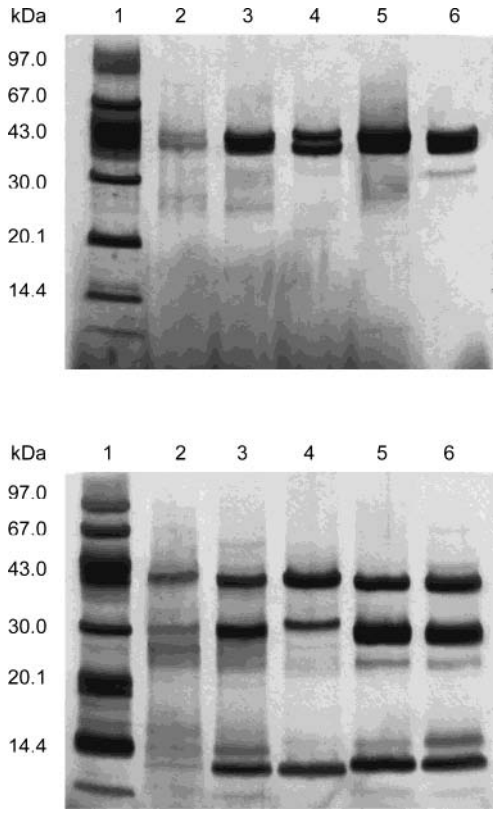

Figure 2. SDS–PAGE (20% w/v) of the different AC–XBS inhibitor fractions under nonreducing (top) and reducing conditions (5% (v/v) 2-mercaptoethanol) (bottom). Proteins were stained with silver. The sizes of the markers are indicated at the side of each gel. Lane 1, low MM markers; lane 2, durum wheat AC–XBS11; lane 3, durum wheat AC–XBS12; lane 4, barley AC–XBS12; lane 5, rye AC–XBS11; and lane 6, rye AC–XBS12.

Only very low amounts of protein and little if any inhibition activity against XBS or XTL2 were found in the corn, rice, oats, and buckwheat AC−XBS fractions. This is an indication for very low levels or even the absence of protein inhibitors, which specifically interact with XBS in these cereals.

Gel Electrophoresis. SDS−PAGE analysis of the durum wheat, barley, and rye AC−XBS inhibitor fractions under

Table 2. Endoxylanase Inhibition Activities of the Different AC–XBS Inhibitor Fractions (0, No Inhibition; +, Weak Inhibition; ++, Strong Inhibition)

		inhibition activity against						
cereal	fraction	XAN[a]	XBS[a]	XTL2[a]	XTL3[a]	XTV[a]	XAA[b]	XANid[b]
durum wheat	AC–XBS11	+	++	+	++	++	0	0
	AC–XBS12	++	++	++	++	++	0	0
barley	AC–XBS12	++	++	++	++	++	0	0
rye	AC–XBS11	++	++	++	++	++	0	0
	AC–XBS12	++	++	++	++	++	0	0

[a] Family 11 endoxylanase. [b] Family 10 endoxylanase.

nonreducing and reducing conditions (**Figure 2**) showed profiles similar to those obtained for the TAXI type inhibitors (*12*, *14*−*16*). Under nonreducing conditions, the inhibitors migrated as double protein bands of ca. 40 kDa, corresponding to the two molecular forms described for this inhibitor family (*9*, *15*). In the presence of 2-mercaptoethanol, the heterodimeric form disappeared and gave rise to two new protein bands of ca. 30 and ca. 10 kDa. In the case of the durum wheat AC−XBS11 fraction, the latter peptide band is not clearly visible, probably due to the low amount of inhibitor applied on the gel.

The different TAXI type inhibitors were basic proteins, as revealed by IEF. Their pI values were ca. 8.5 for the durum wheat and at least 9.0 for the barley and rye inhibitors (results not shown). The latter is in agreement with those found in earlier studies (*15*, *16*).

Inhibition Activity against Endoxylanases. Samples of the different AC−XBS inhibitor fractions, corresponding to 1.5−4 μg of inhibitor (durum wheat) and 5.5−9 μg of inhibitor (barley, rye), were assayed for their abilities to reduce the activities of a number of family 10 and family 11 endoxylanases. In general, family 11 enzymes were affected by all of the inhibitor fractions tested, while family 10 enzymes were insensitive to these fractions (**Table 2**). These findings are similar to those obtained for TAXI I from wheat (*14*, *26*) and confirm the specificities of HVXI and the SCXIs, the barley and rye TAXI type inhibitors, respectively.

Presumably, enzyme inhibitors contribute to plant defense mechanisms and/or possibly intervene in the complex regulation of plant metabolic processes. In this respect, a database search revealed that fungal and bacterial endoxylanases occur in glycosyl hydrolase family 10 as well as in family 11, while the gene sequences of the plant endoxylanases (from *Arabidopsis thaliana*, barley, wheat, rice, and maize) identified to date are exclusively classified in family 10 (*27*, *28*). Therefore, taken together with other data from our group (*14*−*16*, *22*, *26*), the observed specificity of the TAXI type inhibitors, which discriminate between the two endoxylanase families, probably indicates that inhibitors belonging to this family do not regulate metabolic processes in cereals.

Furthermore, XAN and XTL2 were significantly less inhibited by the durum wheat AC−XBS11 inhibitor fraction than the other family 11 endoxylanases were. A similar specificity was reported for TAXI II from wheat (*14*, *26*), indicating that the durum wheat AC−XBS11 inhibitor fraction predominantly consists of a TAXI II like endoxylanase inhibitor(s). In this respect, we noticed that in general, the endoxylanases, which were not affected by the TAXI II inhibitors from wheat (*14*, *26*) and durum wheat, have pI values of 3.5−5.5. In this respect, family 11 endoxylanases have been divided into acidic and alkaline enzymes based on their pI values (*29*). In addition, amino acid sequence alignment of 82 family 11 endoxylanases

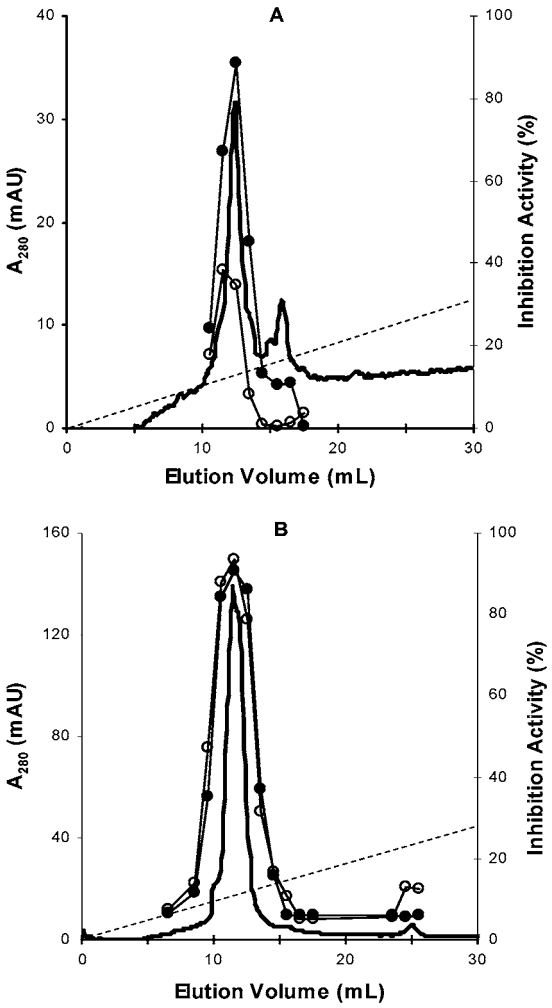

Figure 3. CEC of durum wheat AC–XBS11 (**A**) and AC–XBS12 (**B**) fractions on a MonoS column at pH 6.0, showing the UV absorbance (—), the NaCl gradient (- - -) (0–0.5 M in 100 mL), and the inhibition activity against *A. niger* (XAN) (○) and *B. subtilis* (XBS) (●) endoxylanases.

revealed differences in certain conserved amino acid residues between low and high pI enzymes (*30*). If these structural differences are important for the interaction between enzyme and inhibitor, this suggests that the pI of the endoxylanase may be used to predict the sensitivity to inhibition by TAXI I and TAXI II. However, further experiments are needed to elucidate the different specificity of TAXI I and TAXI II type inhibitors.

Analysis of the Inhibitor Fractions by HR-CEC. Fractionation of the inhibitors in the rye, durum wheat, and barley AC−XBS fractions by HR-CEC, subsequent SDS−PAGE analysis (results not shown), and endoxylanase inhibition assays of samples corresponding to the different protein peaks in the chromatograms revealed that the TAXI type inhibitors of each cereal showed some heterogeneity. In general, different isoinhibitors were eluted with varying elution conditions, indicating a difference in binding strength between the isoforms. Furthermore, each isoinhibitor occurred in a monomeric and a heterodimeric molecular form as demonstrated by SDS−PAGE analyses under reducing and nonreducing conditions (results not shown).

The chromatograms of the separation of the durum wheat AC−XBS11 and AC−XBS12 and the barley AC−XBS12 inhibitor fractions (**Figures 3** and **4A**, respectively) showed a major protein peak, indicating that they mainly contain a single

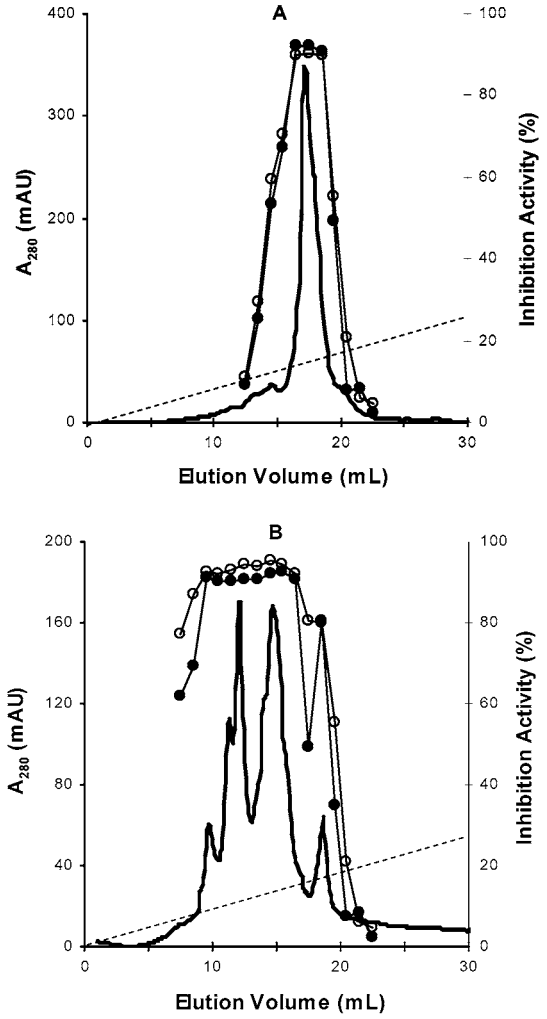

Figure 4. CEC of barley AC–XBS12 (**A**) and rye AC–XBS11 (**B**) inhibitor fractions on a MonoS column at pH 6.0, showing the UV absorbance (—), the NaCl gradient (- - -) (0–0.5 M in 100 mL), and the inhibition activity against *A. niger* (XAN) (○) and *B. subtilis* (XBS) (●) endoxylanases.

isoinhibitor. In barley, this inhibitor corresponds to HVXI, which was previously purified by CEC and GPC (*15*). However, the presence of shoulders on these peaks may be ascribed to additional endoxylanase inhibitors in these fractions. The barley and durum wheat inhibitors were active against XAN and XBS, except for the main inhibitor in the durum wheat AC−XBS11 fraction, which had little effect on the former endoxylanase (**Figures 3** and **4A**). This confirmed the presence of a TAXI II like inhibitor in the durum wheat AC−XBS11 fraction. Therefore, durum wheat, like wheat, contains two different TAXI type inhibitors with varying specificities toward different endoxylanases. These durum wheat inhibitors were named TDXI I and TDXI II.

In contrast to barley and durum wheat, the chromatogram corresponding to the fractionation by CEC of the rye AC−XBS11 inhibitor fraction (**Figure 4B**) displayed several protein peaks and was similar to that of the separation of the rye AC−XBS12 fraction (result not shown) but differed in the relative intensities of the different peaks. However, comparison of the CEC separations of the rye AC−XBS fractions and SCXI I−IV, performed under identical conditions, indicated that in addition to SCXI I−IV, other isoinhibitors were present in the AC fractions. Multiple TAXI isoforms have also been found in wheat (*21*). Because, for the rye inhibitors, small differences in amino acid sequences were found (*16*), the different isoforms at least partially originate from different genes. Inhibition activity measurements showed that similar to barley, the different rye isoinhibitors were active against both XAN and XBS, indicating the absence of a TAXI II like endoxylanase inhibitor in these cereals.

Table 3. Overview of Cereal TAXI Type Endoxylanase Inhibitors

			inhibition activity against		
			family 11 endoxylanases		family 10 endoxyl-
cereals	occurrence	pI	low pI	high pI	anases
	TAXI I type inhibitors[a]				
wheat (TAXI I)[b]	multiple isoforms	≥8.0	strong	strong	none
rye (SCXI)	multiple isoforms	≥9.0	strong	strong	none
durum wheat (TDXI I)	one predominant isoform	∼8.5	strong	strong	none
barley (HVXI)	one predominant isoform	≥9.0	strong	strong	none
oats, rice, buckwheat	no inhibitors purified				
	TAXI II type inhibitors[a]				
wheat (TAXI II)[b]	one predominant isoform	≥9.0	none/weak[c]	strong	none
durum wheat (TDXI II)	one predominant isoform	∼8.5	none/weak[c]	strong	none
rye, barley, oats, rice, buckwheat	no inhibitors purified				

[a] Characterized by monomeric (40 kDa) and dimeric (30 + 10 kDa) molecular forms. [b] See Gebruers et al. (*21*). [c] Except for *Penicillium funiculosum* XYNC, which is strongly inhibited by TAXI II (TDXI II, not determined) (*31*).

CONCLUSIONS

Purification by AC with immobilized XBS and successive characterization of the cereal endoxylanase inhibitor fractions, combined with previous studies on TAXI type inhibitors (*14*−*16*, *21*), provided a more detailed view on the occurrence and properties of these endoxylanase inhibitors in different cereals (**Table 3**). TAXI type endoxylanase inhibitors have been purified from wheat (*14*, *21*), rye, durum wheat, and barley. For the cultivars used in these studies, the highest TAXI type inhibitor levels occur in wheat (ca. 38 ppm) (*21*) and rye (ca. 22 ppm), while lower levels are found in durum wheat (ca. 11 ppm) and barley (ca. 4−5 ppm). In general, all isolated inhibitor fractions were heterogeneous to some extent, comprising several isoforms of the inhibitors. No TAXI type endoxylanase inhibiting proteins were isolated from corn, rice, oats, and buckwheat.

TAXI type endoxylanase inhibitors can be further classified into the TAXI I and TAXI II subfamily with different specificities. TAXI I type inhibitors strongly inhibit both high and low pI family 11 endoxylanases and occur in wheat, rye, durum wheat, and barley. TAXI II type inhibitors are potent inhibitors of high pI family 11 endoxylanases and only poorly or do not inhibit the low pI enzymes. They have been found in wheat and durum wheat but not in rye or barley. Neither TAXI I type nor TAXI II type inhibitors reduce the activity of family 10 endoxylanases (**Table 3**).

ABBREVIATIONS USED

AC, affinity chromatography; BSA, bovine serum albumin; CEC, cation exchange chromatography; GPC, gel permeation chromatography; HR-CEC, high-resolution cation exchange chromatography; HVXI, *Hordeum vulgare* xylanase inhibitor; IEF, isoelectric focusing; InU, inhibition unit; SCXI, *Secale cereale* xylanase inhibitor; TAXI, *Triticum aestivum* xylanase inhibitor; TDXI, *Triticum durum* xylanase inhibitor; XAA, *Aspergillus aculeatus* endoxylanase; XAN, *Aspergillus niger* endoxylanase Xyn1; XANid, *Aspergillus nidulans* endoxylanase;

XBS, *Bacillus subtilis* endoxylanase XynA; XIP, xylanase inhibiting protein; XTL2, *Trichoderma longibrachiatum* endoxylanase; XTL3, *Trichoderma longibrachiatum* endoxylanase; XTV, *Trichoderma viride* endoxylanase.

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